Abstract

Objective: Polycystic ovary syndrome (PCOS) is the most common endocrine disorder of reproductive women. It is affecting from 5-10% of women in reproduction age. The symptoms include infertility, hirsutism and acne, menstrual abnormalities, Obesity and may cause endometrial cancer, cardiovascular disorder and diabetes mellitus. Leptin, the product of the *ob* gene, is a small peptide molecule synthesized by white adipose tissues with an important role in the regulation of fat body and food intake. Although the role of leptin in the control of reproduction is unclear, but it may be involved in the control of ovulation. Radical Oxygen Species play a physiological role throughout ovulation. The antioxidant (Vitamin E and Selenium) have important role in infertility and in PCOS.

Materials and Methods: This study included twenty-five untreated women with clomiphene citrate, twenty-six treated with clomiphene citrate and twenty-four control women matched- body mass index (BMI), each of these groups are subdivided to three subgroups according to menstrual cycle phases. Fasting blood samples were collected. Concentrations level of parameters in serum was analysed (Leptin by Enzyme-linked immosorbent assay (ELISA) which is based on sandwich principle, Estradiol (E_2) by Mini-Vidas which combines a competitive method with a final fluorescent detection (ELFA) Enzyme linked fluorescent assay, Vitamin E by High performance liquid chromatography (HPLC), and Selenium (Se) by flameless atomic absorption spectrophotometer (AAS)).

Results: During the three phases of Menstrual cycle (M.C.), there are a significant difference between follicular and pre-ovulation phase (leptin, estradiol, vitamin E, selenium; $P<0.001$, $P<0.0005$, $P<0.05$, $P<0.01$. respectively) for treated and control groups, while untreated ($P<0.05$, $P<0.005$, N.S., $P<0.01$. respectively). Also there are significant difference between pre-ovulation and luteal phase for estradiol ($P<0.0005$) in control and treated groups. Serum leptin concentrations were not different in subjects with groups of PCOS and controls matched- BMI, and were associated BMI with positive correlation for each group (Untreated, Treated, Control; $r=0.895$, $r=0.847$, $r=0.823$ respectively). While serum estradiol and vitamin E have significant between PCOS groups and controls in pre-ovulation period ($P<0.0005$), and serum estradiol concentrations positive correlation with BMI only in untreated PCOS($r=0.531$), also serum estradiol concentrations have positive correlation with leptin in follicular phase (Untreated, Treated, Control; $r=0.915$, $r=0.898$, $r=0.786$ respectively). Selenium have highly significant difference between PCOS groups and control in each phase of M.C. ($P<0.000005$) and there are no association between selenium and vitamin E with BMI.

Conclusion: The results indicated that leptin concentrations do not differ significantly between PCOS patients and control with clear positive correlated with BMI. Leptin have a role in follicular growth by their relation with estradiol at which indirect effect to selenium. Vitamin E has a role in ovulation. In untreated PCOS lower level of Selenium and vitamin E suspecting of oxidative stress is occurred.

الخلاصة

الموضوع: أن مرض المبيض المتعدد الاكياس هو حالة مرضيه شائعة في الاناث خلال سنوات الاخصاب و بنسبة(٥-١٠ %) و يعتبر أحد اسباب العقم عند النساء. و من أعراضه: عدم القدره على الأنجاب، أنبات الشعر، عدم انتظام الدورة الشهرية ، السمنة ، سرطان البطانة الداخلية للرحم ، أمراض القلب، و مرض السكر. أن اسباب المرض غير معروفة.

بروتين السمنه اللبتين المتكون في الانسجة الشحميه و الذي له دور مهم في تنظيم دهون المخزونه في الجسم و مقدار الطعام المتناول. على الرغم من أن دور اللبتين في التكاثر غير معروف و لكن من المحتمل ان يكون له دور في عملية الاباضة.

ان جذر الاوكسجين الحر له دور فسيولوجي خلال عملية الاباضه و المضادات للأكسدة (السيلينيوم و الفيتامين إي) لهما دور مهم في العقم، و لكن دورهما في هذا المرض غير معروف.

الطريقة و المواد: هذه الدراسة تتضمن خمسة و عشرون مريضه غير معالجه بعقارالكلوميفين ستريت، و ستة وعشرون مريضه معالجة بعقار الكلوميفين ستريت، بالمقارنة مع أربعة و عشرون من النساء الاصحاء.

مستوى تراكيزالمتغيرات التي قيست في مصل الدم كل من (اللبتين بواسطة الفحص المناعي المرتبط بالانزيم ، الاسترادیول بواسطة جهاز المني فايدص، فيتامين إي بواسطة كرموتوكرافيا السائل،السيلينيوم بأستخدام تقنية مطيافية الامتصاص الذري عديم اللهب).

النتائج: خلال أطوارالدورة الشهرية الثلاث يوجد اختلاف معنوي بين طور الحويصلي و طورقبل التبويض للمجموعة المعالجة و الأصحاء (اللبتين، الأسترادايول، الفيتامين إي، السيلينيوم؛ $P<0.001$, $P<0.01, P<0.05, P<0.0005$ بالترتيب). بينما المجاميع الغيرمعالجه لايوجد هذا الاختلاف المعنوي للفيتامين اي ($P<0.01, P<0.005, P<0.05$ بالترتيب).

و يوجد أيضا اختلاف معنوي للاسترادايول بين طور قبل التبويض و طور الجسم الاصفر للمرضى المعالجين و الاصحاء ($P<0.0005$) . أن مستوى تراكيز اللبتين لايختلف اختلافا معنوي بين المرضى و الاصحاء المتساوين في كتلةالجسم والذي له علاقة طردية مع كتلة الجسم في كل المجاميع(الغير معالجه، المعالجه، و الأصحاء؛ $r=0.823, r=0.847, r=0.895$ بالترتيب) بينما الاسترادايول و

فيتامين إي يوجد في تركيزهما اختلاف معنوي بين المجموعتين المرضى والأصحاء في فترة قبل التبويض ($P<0.0005$) مع وجود علاقة طرديه بين الاستراديول و كتلة الجسم في المجموعة الغير معالجة($r = 0.531$)ومع اللبتين في الطور الحويصلي لكل المجاميع المدروسه(الغير معالجه، المعالجه، و الاصحاء؛ $r= 0.786, r= 0.898, r= 0.915$ بالترتيب). يوجد اختلاف معنوي بين المرضى و الاصحاء في تراكيز السيلينيوم في جميع أطوار الدورة الرحميه ($P<0.000005$) و يوجد علاقة له مع كتلة الجسم و كذلك بالنسبة الى فيتامين إي.

الأستنتاج: النتائج أظهرت، أن تراكيز اللبتين ليس له أختلاف معنوي بين المرضى والأصحاء المساويين لهم بكتلة الجسم مع وجود علاقة قوية له مع كتلة الجسم.ان اللبتين له دور في نمو الحويصله من خلال علاقته بالأستراديول و الذي له تأثير غير مباشرعلى السيلينوم. فيتامين إي له دور في عملية التبويض، كذلك أن أنخفاض مستواه مع السيلينيوم في مجموعة المرضى الغيرمعالجه يشكك في تكون جذر حر.

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Chapter One

Introduction & Literature review

1.1. Polycystic Ovary Syndrome (PCOS)

Is the most common endocrine disorder of women in reproductive age, affecting from 5-10% of women in this age group [1, 2].

It was first described in 1935 by Stein and Leventhal, and for many years PCOS was known as the Stein-Leventhal syndrome [3, 4].

1.1.1. Definition

PCOS is clinically defined as oligomenorrhea associated with hyperandrogenism [5].

It has been described poetically as "the thief of women hood" because women with (PCOS) seek medical attention for infertility and hirsutism [6].

1.1.2. Feature of PCOS

1.1.2.1. Clinical feature: [7]

1. Menstrual abnormalities including oligomenorrhea (irregular periods), amenorrhea (absent periods).
2. Hirsutism (excessive hair growth on face, chest, abdomen ….. etc).
3. Androgenic alopecia (hair loss).
4. Acne.
5. Infertility or reduced fertility.

6. Polycystic ovaries are characterized by the accumulation of small follicles 4-9 mm in diameter with hypertrophied theca interna layers [1, 7]. Figure (1.1)

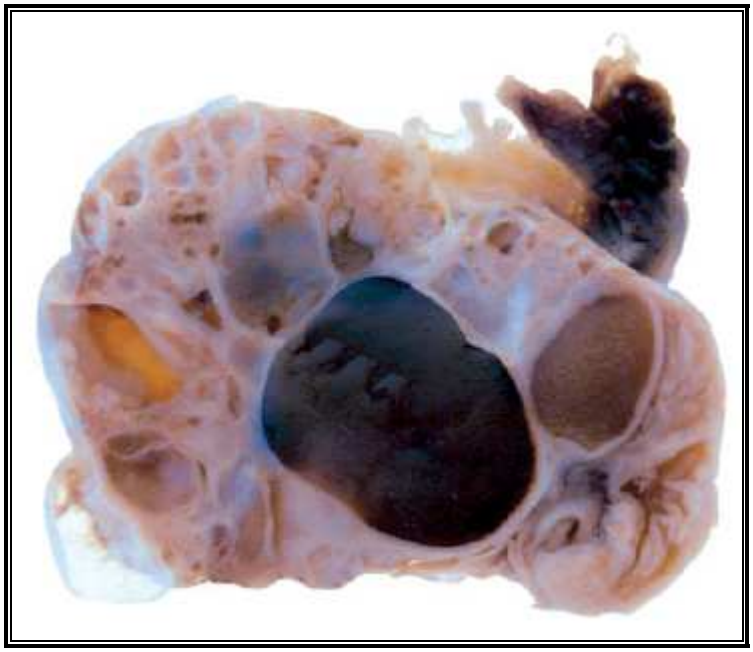


Fig. (1.1): Stained longitudinal section of polycystic ovary showing numerous small peripheral follicles.
[Lakhani *et al.* 2002] (8)

1.1.2.2. Endocrine Feature: [4]

1. LH/FSH ratio which is normally 1:1 but in PCOS is often 3:1.

2. Androgens typically testosterone and androstenedione are elevated but this is not universal finding. Sex-hormones binding globulin (SHBG) level which may be reduced particularly in obese women with PCOS, this leads to elevated level of active androgens.

3. Insulin serum levels may be elevated.

4. Some women have hyperprolactineima.

1.1.2.3. Metabolic Feature: [9]

The complicate of PCOS are:

1. Elevated cardiovascular risk factor.

2. Type 2 diabetes.

3. Obesity.

4. Endometrial hyperplasia.

5. Endometrial cancer.

1.1.3. Ultrasound Features:

The finding of polycystic ovaries on ultrasound, which is characterized by an increased number (12 or more) of fluid-filled sacs in each ovary, containing an immature egg measuring up to 9 mm in diameter, and/or increased ovarian volume [10]. As show in Figure (1.2)

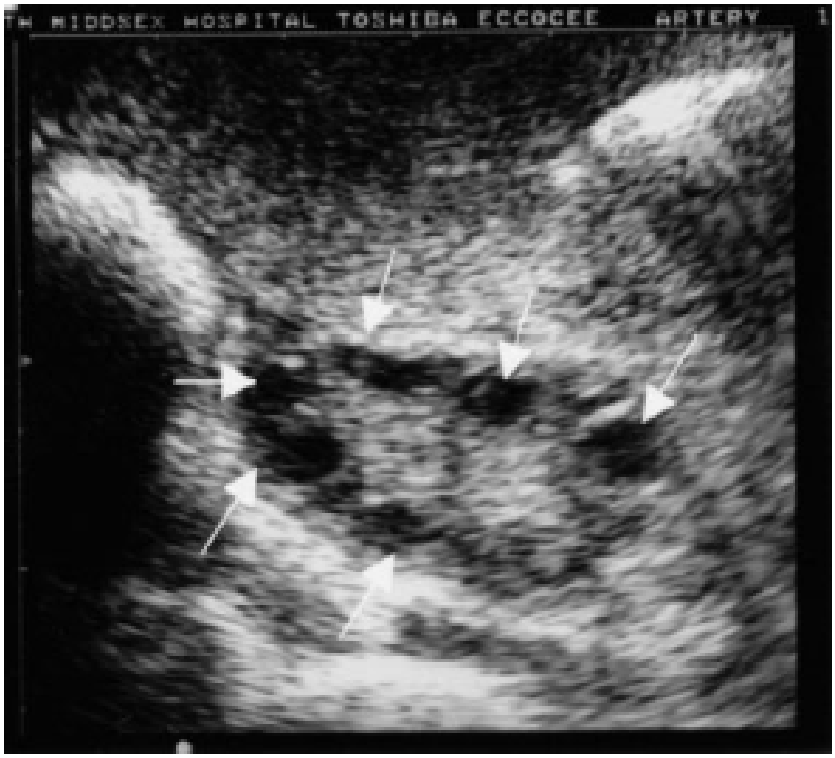


Fig. (1.2): Ultrasound of Polycystic ovary showing peripheral distribution of follicles (arrows).
[Lakhani *et al.* 2002] (8)

1.2. Hypothalamus-Pituitary-Ovarian axis

The luteinizing releasing hormone is produced by the hypothalamus in pulses. It control gonadotropin hormones (Follicle stimulating hormone FSH and Luteinizing hormone LH) synthesis and release, these hormones are secreted by the anterior lobe of the pituitary gland.

FSH stimulates ovarian follicle growth which produces estradiol during the follicular phase of the ovarian cycle then estrogen stimulates endometrial cell.

A preovulatory estrogen peak is followed by LH peak which causing follicular maturation and ovulation. As show in Figure (1.3). LH triggers rupture of the dominant follicle to release the oocyte (ovum). It usually occurs at about 14 day before the next period.

With ovulation the second half (Luteal phase) of the cycle begins. After ovulation the corpus luteum is formed in the ovary at site of the empty ruptured follicle stimulated by LH. The corpus luteum produces both estrogen and progesterone.

Estrogens, progesterone and androgen hormones which are normally produced by the ovary are chemically interchangeable by enzymes [(11)].

1.2.1. Estrogens

Estrogens are ovarian sex hormones that mainly promote proliferation and growth of specific cells in the body that are responsible for the development of most secondary sexual characteristics of the female.

Only three estrogens are present in significant quantities in the plasma of the human female: β-estradiol, estrone, and estriol.

Estrogens was secreted by the ovaries is β-estradiol, which is more potency among the other. Small amounts of estrone are also secreted, but most of this is formed in the peripheral tissues from androgens secreted by the adrenal cortices and by ovarian thecal cells [(12)].

There is extra-ovarian estrogen formation in adipose tissue and skin which is considered a major source for circulating E2 in the postmenopausal period or during ovarian suppression [(13)].

The concentration of plasma estradiol depends on the age and time of menstrual cycle in women [(14)]. Younger women tend to have higher levels of estradiol, with plasma concentrations ranging from 50 to 500 pg/ml whereas postmenopausal women would be expected to have estradiol values of approximately 10–20 pg/ml. Estradiol levels vary during the normal menstrual cycle of women reaching peak levels during mid-cycle [(15)]. Figure (1.3).

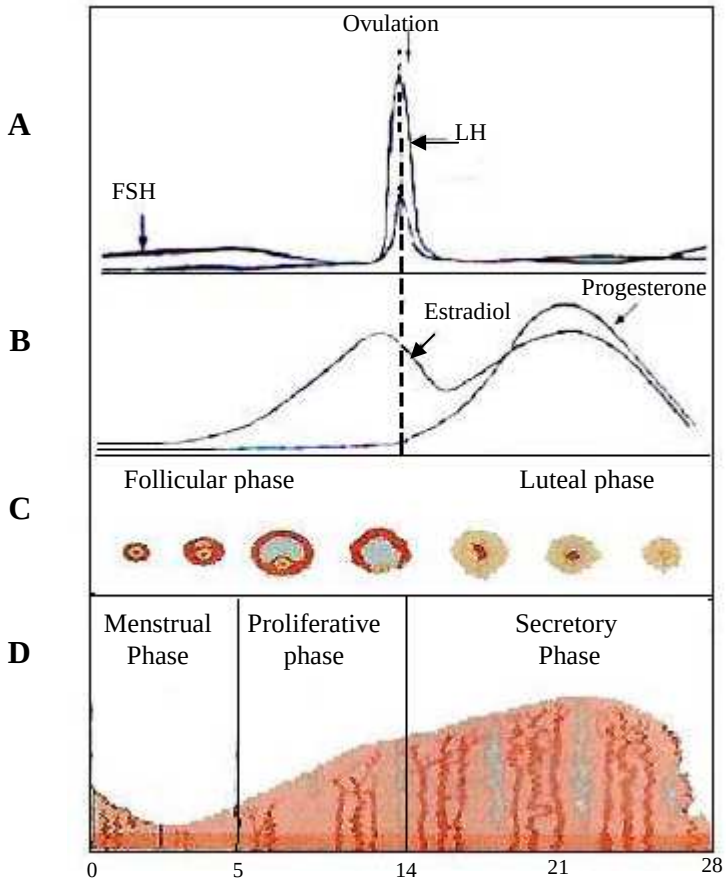


Fig. (1.3): Levels of hormones in menstrual cycle

A-Fluctuation of gonagotropin levels.
B-Flutuation of ovarian hormone levels.
C-Ovarian cycle.
D-Menstrual cycle (uterine cycle).

[Hacker *et al.* 2004] (11)

Estrogens secretion is usually abnormal in PCOS. Estradiol levels may be low to normal, and in the anovulatory cycle there is tonic production without the increase before ovulation or in the midluteal phase as in normal women [16, 17] while Estrone levels increase due to extraglandular conversion of androstenedione in adipose tissue [18].

1.3. Pathogenesis of PCOS

No single etiologic factor fully accounts for the wide spectrum of metabolic abnormalities seen in PCOS [6].

Recent discoveries have linked PCOS to a general disorder of insulin metabolism [19].

Insulin resistance is worsened by the coexistence of obesity, which is also increased in the PCOS population [3]. More than 40 % of PCOS patients are obese [20, 21].

The increased insulin resistance leads to excessively high level of insulin as a compensatory mechanism although the ovaries remain sensitive to insulin [9]. This excessive high insulin stimulates excess androgen production by ovarian theca and decreased sex hormone binding globulin (SHBG) from hyperandrogenism leading to further increase in free testosterone levels and, ultimately, to hirsutism and acne [6].

In the ovary, the inability to produce estradiol because of the decreased activity of FSH-related aromatase attenuates the development of ovulatory follicles, thereby causing the development of a large number of follicles in various stages of arrested maturation, giving arise to typically picture of polycystic ovary [18].

Furthermore insulin amplifies the LH response of granulose cells, thereby causing an abnormal differentiation of these cells, premature arrest of follicular growth, and so, anovulation.

Estrone level increase due to extraglandular conversion of androstenedione in adipose tissue, which further stimulate LH and inhibits FSH secretion, causing

stimulation and hyperplasia of ovarian stroma and theca cells, leading to increased androgens. This provides more substrate for extraglandular aromatizatin of androgens to estrogens and perpetuates the cycle [18].

1.4. Leptin

In recent years it has been shown that a dipocytes are secretory cells that produce a variety of proteins with hormonal-type function, which collectively have been called a dipocytokines. One of them discovered was ***leptin*** [22].

1.4.1. Definition

Leptin is a small peptide product of the *ob* gene. It is a 16 KDa non-glycosylated polypeptide of 146 amino acid discovered by Zhang *et al.* in 1994. The precursor form of leptin contain 167 amino acid is activated by cleavage of a 21 amino acid residue [22, 23]. The word leptin is derived from the Greek word 'leptos' that means thin [24].

1.4.2. Secretion and action

Leptin is synthesized and secreted from white adipocytes into blood and is transported into the brain via a saturable system [25] to reduces the production of neuropeptide Y (NPY) from the arcuate nucleus of the hypothalamus [26] which under normal condition increases food intake in animals, and after chronic administration produces obesity [27]. Then result in a reduction in food intake, an increase in energy expenditure and increased physical activity.

Additionally, leptin (and perhaps other factors) acts in a negative feed back loop to inhibit further expression of the leptin gene [25].

Extensive research on leptin over the last years has shown that leptin is not only a messenger of the amount of energy stores to the brain [28], but also a hormone / cytokine for diverse physiological processes, such as inflammation, angiogenesis, hematopoiesis, immune function, and reproduction [29].

1.4.3. Leptin and Hypothalamic-Pituitary-Gonadol (HPG) axis

It is well established that alterations in nutritional status and/or energy reserves can disrupt the HPG axis resulting in reduced fertility.

Accumulating evidence suggests that leptin may serve as this critical link between adipose stores and hypothalamic enters that control the gonadal axis in rodents [30].

Leptin receptors (Ob-Rs) have been identified in the hypothalamus, gonadotrope cells of the anterior pituitary, granulosa, theca, and interstitial cells of ovary, endometrium [31], and leydig cells [32].

In 2002 Mancini and Domenico de Aloysio found this multilocal expression of leptin, as well as the presence of Ob-Rs at all levels of the hypothalamus-pituitary-Gonadal (HPG) axis, implies that the nutritional / leptin regulation of reproduction involves a complex network of interactions to regulate the HPG axis in a paracrine and/or endocrine way [30].

At level of hypothalamus, leptin was reducing the production of neuropeptide Y (NPY) [26], which has inhibitor effect on gonadotropin secretion [33-38] then at level pituitary, leptin have stimulatory effect on LH release [39] and at level of ovary, leptin increased ovarian estrogen production [40]. In addition, estradiol concentration could be influence leptin synthesis [24, 41], as show in Figure (1.5).

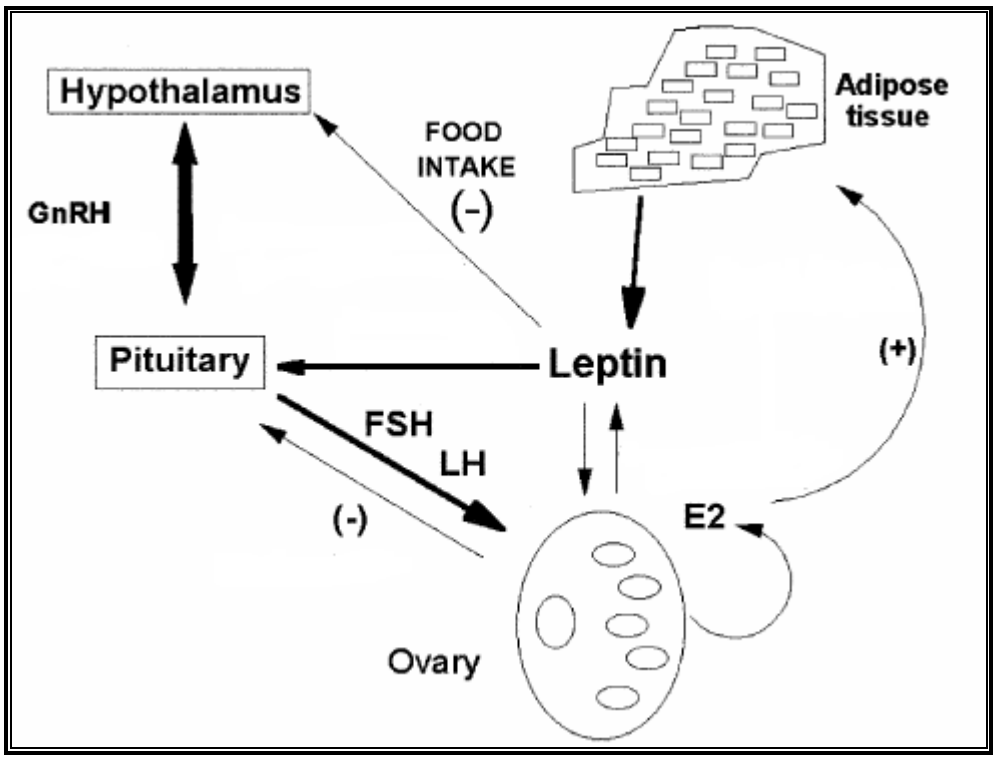


Fig. (1.4): Represents leptin as regulator of HPG axis.
(+): Stimulatory effect, (-); inhibitory effect.
[González *et al.* 2000] (42)

1.4.3.1. Leptin level during the menstrual cycle

The importance of leptin for normal fertility has been demonstrated in *ob/ob* mice lacking leptin. In these animals, administration of leptin induces an increase both in LH concentrations and in ovarian and uterine weights [(40)].

In humans a critical blood leptin concentration appears to be necessary to trigger the onset of ovarian activity [(43)]. Furthermore, circulating leptin concentrations appear to be better predictors of menstrual function than body mass index (BMI), fat mass or percentage body fat [(44)].

At the level of the ovary, human ovarian follicles have leptin receptors [(45)], their follicular cells express leptin mRNA at the time of dominant follicle selection [(46)], and granulose cells have been shown to secrete leptin [(47)],indicating a likely role for leptin in normal ovarian physiology.

Many studies have demonstrated that leptin has physiological fluctuations during the menstrual cycle, and its concentrations were significantly lower in the early follicular phase [24, 41, 48-52]. However, other investigators have reported a different pattern for serum leptin concentrations during the menstrual cycle; leptin concentrations were found to be similar during the early and late folliclar and late secretory phases [53]. While, other study has shown that leptin concentrations started to in the late follicular phase, reaching a plateau at the mid cycle gonadotropin surge and the early luteal phase [54].

All these studies indicating that leptin plays a role in ovulation.

1.4.3.2. Leptin and PCOS

Evidence has been provided since the early 1970s that fat mass may directly affect ovulation and fertility [55]. More recent evidence suggests that leptin also has direct regulatory actions on the developing follicle. The presence of leptin receptors on follicular cells, including oocytes, and early preimplantation embryos suggests that leptin may play a direct physiologic role in follicular maturation, oocyte development, and early cleavage [56].

Some women with PCOS are obese. With the possible involvement of leptin in the development of human obesity, as in animals, several investigators have attempted to examine the role of this protein in the pathophysiology of PCOS [57].

The first study, in which serum leptin concentrations were measured in women with PCOS, was published in 1996. In that study, 29% of the women with PCOS had serum leptin values above the 99% prediction interval for their BMI. These women with insulin resistance had serum leptin values higher than controls, while a positive correlation was found between leptin concentrations and insulin sensitivity [57]. Since insulin is known to increase leptin mRNA in adipocytes, [58] it is possible that insulin may stimulate the secretion of leptin and, therefore, leptin may participate in certain cases of PCOS [57].

Subsequent studies, however, have not confirmed these data. In these studies, serum leptin concentrations did not differ significantly between women with PCOS and normal controls matched BMI [59-62].

Although leptin metabolism has been related to PCOS, its role in pathogenesis of PCOS is unknown at the present time.

1.5. Free radicals

Since reproductive and developmental process accompany dynamic changes in metabolism and energy consumption, byproducts are also generated on an extraordinary scale. Among such byproducts, reactive oxygen species (ROS), which are inevitably generated during the physiological process of oxygen consumption, the levels of which are enhanced under some pathological conditions [63].

1.5.1. Oxidants

Reactive Oxygen species (ROS) are oxygen-derived molecules which are a class of powerful oxidants in the human body.

ROS include: superoxide anion $O_2^{\cdot -}$, hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$).

Aerobic environment is constant source of ROS through in vivo mechanisms such as electron leakage during biologic oxidations, and by physical activation of oxygen by external agents such as irradiations, e.g. UV sunlight [64].

Oxidants are versatile molecules well known for their ability to react with virtually all cellular components. When they are present above a critical threshold, they can induce significant structural and functional cell damage. However, concentrations of oxygen radicals below this threshold have been shown to play a physiological role in the processes of fertilization and oocyte maturation [65, 66].

Role of Reactive Oxygen Species (ROS) during Ovulation

Reactive Oxygen Species (ROS) appears to have physiological role in female reproductive tract in many different processes such as: oocytes maturation, fertilization, luteal reprogression, and endometrial shedding [65, 67].

Ovary is a metabolically active organ and, hence, is under a variety of stresses continuously. ROS play a physiological role during ovulation that is similar in some respects to inflammation [68, 69].

Since ROS is generated during inflammatory process, it is reasonably hypothesized that ROS is released in connection with follicle rupture and is involved in the process [70], as show in Figure (1.6).

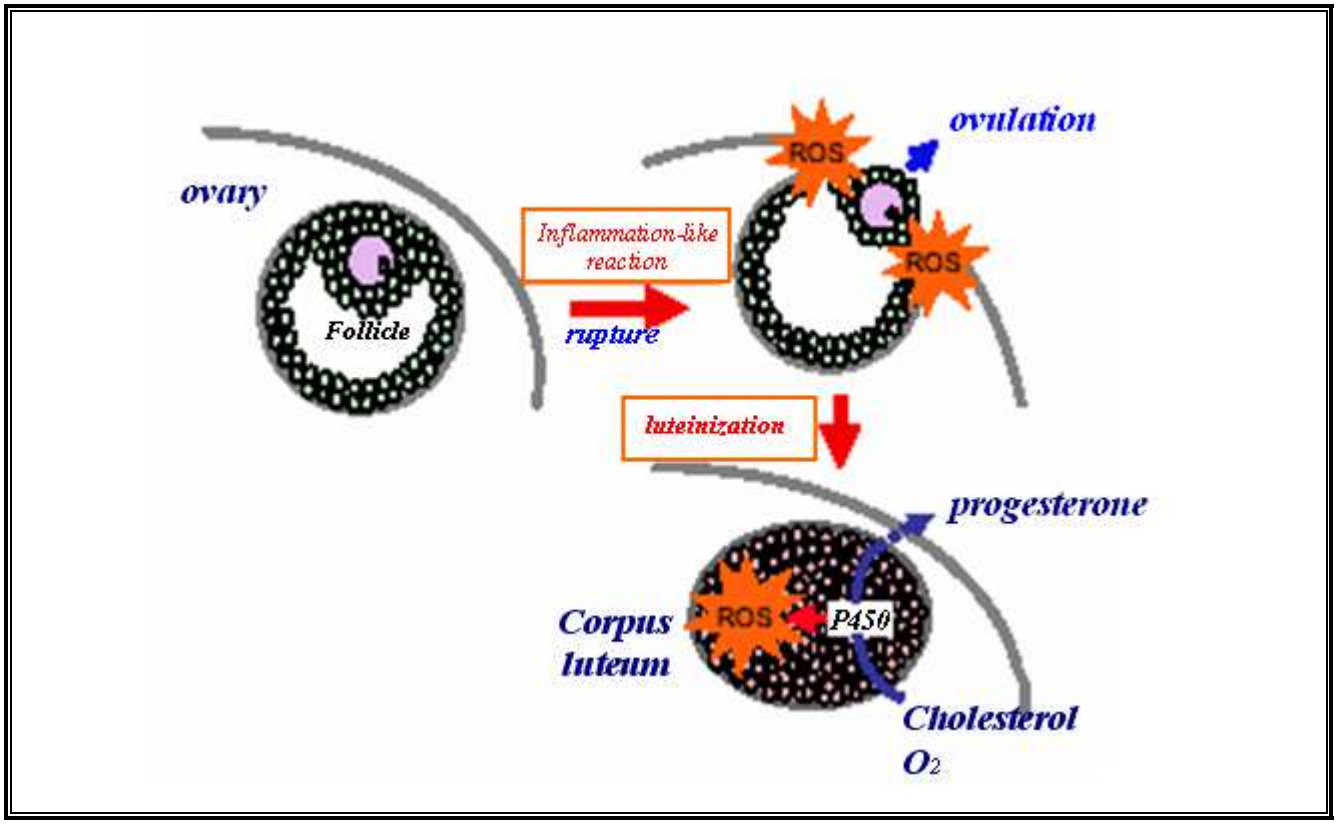


Fig. (1.5): The Role of Reactive Oxygen Species (ROS) during ovulation process
[Fujii *et al.* 2005] (70)

The source of ROS appears to be inflammatory cells, such as macrophages and neutrophils, as they are present in ovary at ovulation [71-73].

The steroidogenic process that inevitably produces ROS as by products, ROS levels in the corpus luteum actually increase during the regression phase [74-79]. ROS and related compounds may function as intracellular regulators of steroidogenesis and progesterone release in the corpus luteum [76, 80-82].

1.5.2. Antioxidants

Antioxidants are substances that donate electrons to oxygen-based free radicals forcing it to change from the excited to the ground state. Resulting neutralization and stabilization of ROS and prevent oxidative stress, for this reason it represent the productive mechanisms used by cells against oxidants [83].

In a healthy body, pro-oxidants and antioxidants maintain a ratio and a shift in this ratio towards pro-oxidants gives rise to oxidative stress. This oxidative stress may be either mild or severe depending on the extent of shift.

Whenever ROS levels become pathologically elevated, antioxidants begin to work and help minimize the oxidative damage, repair it or prevent it altogether [84].

Many studies reported that presence of oxidant and antioxidant systems in various female reproductive tissues [85-89].

Sikka SC in 2004 classified the types of biological antioxidants into [90]:

1. Enzymatic antioxidants such as catalase, superoxide dismutase and glutathione peroxidase/ reductase
2. Non-Enzymatic antioxidants such as Vitamin C, Vitamin E, Vitamin A, pyruvate and glutathione.

Antioxidant status may be one determinant of reproductive function in dairy cattle. Administration of vitamin E or the combination of vitamin E and selenium has been reported to reduce the incidence of postpartum reproductive disorders such as retained fetal membranes, metritis, and cystic ovaries [91-93] and to improve fertility [93].

1.5.2.1. Vitamin E

A factor in vegetable oils that restored fertility to rats was isolated in the early 1920s as vitamin E; later it was given the generic name tocopherol and was shown to include several biologically active isomers shown in figure (1.6)

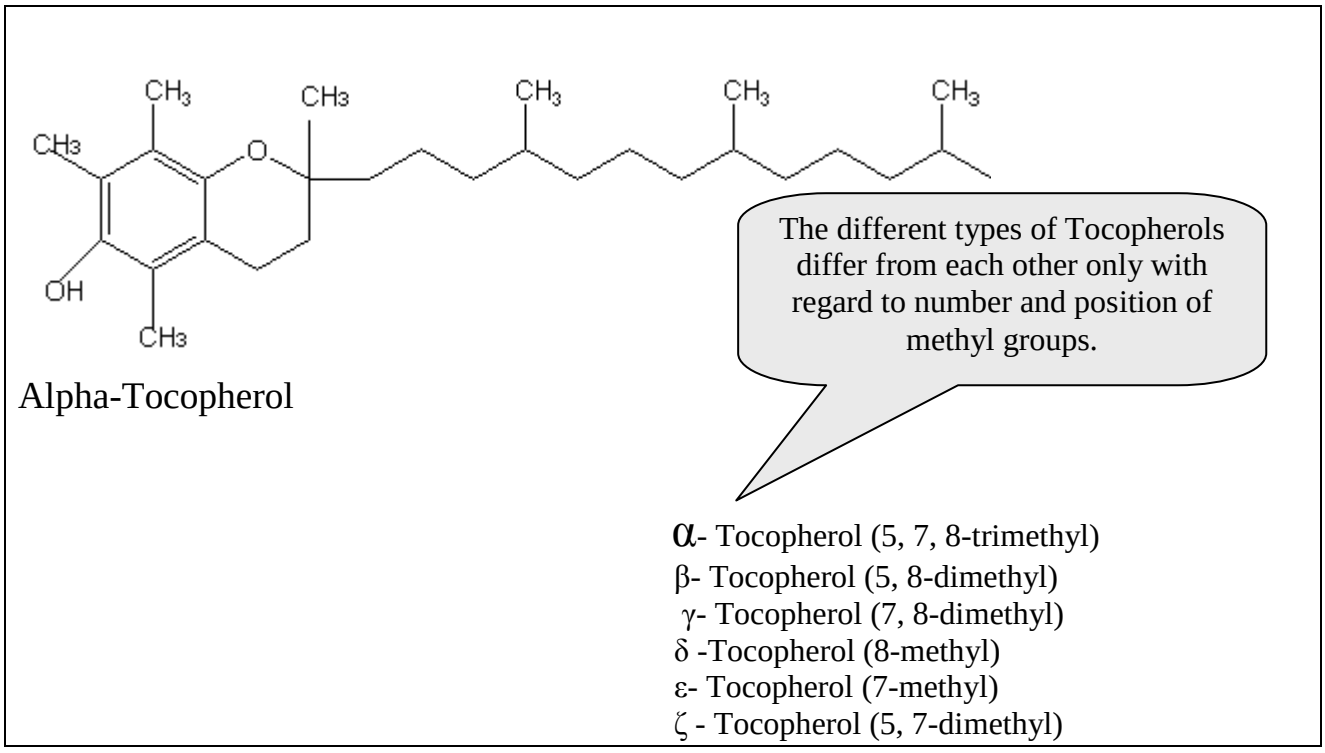


Fig. (1.6): Vitamin E isomers.
[Raju & Madala, 2005] (94)

α- Tocopherol is the predominant isomer in plasma and is the most potent isomer by current biological assays.

The word tocopherol is of Greek derivation, meaning oil that "brings forth in childbirth", but the fertility role of these compounds is still questionable.

Dietary sources of tocopherols include vegetable oils, fresh leafy vegetables, egg yolk, legumes, peanuts, and margarine [(95)].

Concentration of α-tocopherol in serum of 5 – 20 µg/mL for adults and children of twelve years or older and values of 3 – 15 µg/mL for children under twelve years [(96)].

1.5.2.1.1. Metabolism

Vitamin E is absorbed from the intestines, packaged in chylomicrons. It is delivered to the tissues via chylomicron transport and then to the liver through chylomicron remnant uptake.

The liver can export vitamin E in vary low density lipid (VLDLs). Due to its lipophilic nature; vitamin E accumulates in cellular membranes; adipose tissue and other circulating lipoproteins. The major site of vitamin E storage is in adipose tissue [(94)].

1.5.2.1.2 Function

The main function of vitamin E is as a chain-breaking, free radical trapping antioxidant in cell membranes and plasma lipoproteins. It reacts with lipid peroxide radicals formed by preoxidation of polyunsaturated fatty acids before they can establish a chain reaction. The tocopheroxyl radical product is relatively unreactive and ultimately forms nonradical compounds (Figure 1.7) [(97)].

1.5.2.1.3. Vitamin E deficiency

No major disease states have been found to be associated with vitamin E deficiency due to adequate levels in the average diet. The major symptom of vitamin E deficiency in humans is an increase in red blood cell fragility. Since vitamin E is absorbed from the intestines in chylomicrons, any fat malabsorption diseases can lead to deficiencies in vitamin E intake [(94)].

Deficiency of vitamin E affect in infertility because of Vitamin E prevents loss of spermatogenesis in males but it failure to retain zygotes in female rats [(98)].

Also deficiencies of vitamin E lead to alterations in the synthesis of steroid hormones and the prostaglandins [(92, 99-101)].

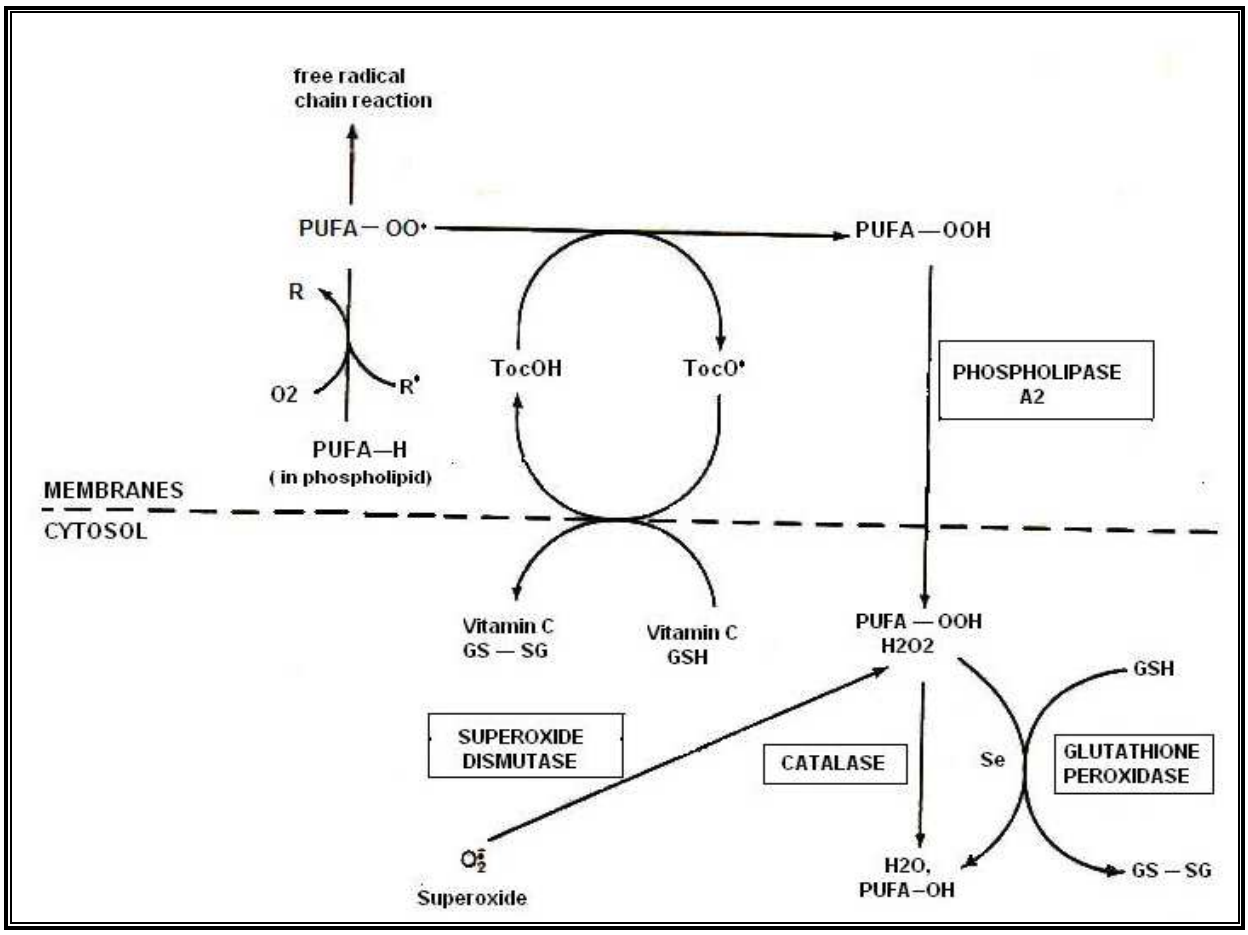


Fig. (1.7): Antioxidant systems of the cell.

(R·, free radical; PUFA-OO·, peroxyl free radical of polyunsaturated fatty acid in membrane phospholipid; PUFA-OOH, hydroperoxy polyunsaturated fatty acid in membrane phospholipid released as hydroperoxy free fatty acid into cytosol by the action of phospholipase A2 ; PUFA-OH, hydroxy polyunsaturated fattyacid; TocOH, vitamin E(α-tocopherol);TocO·, free radical of α-tocopherol; Se, selenium; GSH, reduced glutathione; GS-SG, oxidized glutathione).

[Murray *et al*. 2003] (97)

1.5.2.2. Selenium (Se)

Selenium is a trace element essential in small amounts, but it can be toxic in larger amounts. Levels in the serum are mainly dependent on the amount of Se in the e diet, which is a function of Se content of the region [(102)] .

The majority of median serum selenium concentrations in the world range from 80-120 µg/L [(103)].

Se in food is primarily present in an organic form as two modified amino acids: selenomethionine (synthesized by plants) and selenocysteine (synthesized by animals)[104].

A sex hormone effect on selenium utilization has also been indicated in humans since serum selenium concentration in boys decreases during sexual maturation, whereas this change does not occur in girls [105]. Sex differences also appear to influence the distribution of selenium and GPx activity, since erythrocyte GPx activity was shown to be greater in females than in male [106].

1.5.2.2.1 Metabolism

Se is an integral part of more than about 30 known proteins. These proteins are called Selenium-containing proteins or selenoproteins [107].

Selenoproteins are enzymatically digested in small intestine to yield amino acids, oligopeptides, L-selenomethionine and L-selenocystine.

After absorption from the small intestine, via a similar mechanism to that of L-methionine, selenomethionine is transported to the liver, where a fraction is extracted by the hepatocytes. The remaining amount is transported by circulation to the various tissues of the body [108, 109].

1.5.2.2.2. Functions of Se [110]

1. It is a cofactor for glutathione peroxidase so it is a potent antioxidant.
2. It converts thyroxine to tri-iodothyroxine.
3. It is involved in the inflammatory response.
4. It enhances the antioxidant effect of vitamin E.
5. Binds heavy metal such as Cd and mercury.

1.5.2.2.3. Selenium deficiency

Selenium deficiency in females results in infertility, abortions and retention of the placenta [111].

Also a significant depletion of Se in follicular fluid of women with unexplained infertility has been described in humans [112].

In vitro studies using bovine granulosa cells obtained from different-sized follicles found that Se significantly stimulated the proliferation of cells from small follicles and augmented the stimulatory effects of gonadotrophins in the same cells. Se also enhanced oestradiol production [113]. The relevance of these observations to humans is not known [114].

1.6. Clomiphene Citrate (CC)

CC is a triphenylethelene derivative. It was first synthesized in 1956 and was reported to be effective in inducing ovulation by Green blatt *et al.* in 1961 and it remains the most commonly used drug in the treatment of infertility [115].

1.6.1. Chemistry

CC is racemic mixture of En and Zu isomers [116], its chemical name is 1-[*p*(β-diethylaminoethoxy)phenyl]-1,2-diphenylchloroethylene [117] ,as show in figure(1.8) below.

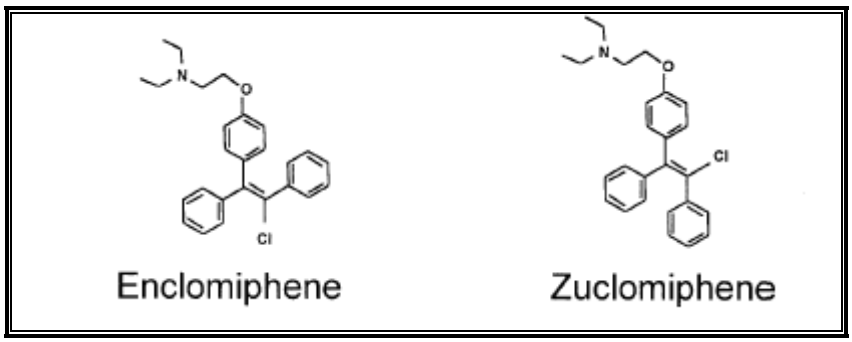


Fig. (1.8): Isomers of Clomiphene.
[Goldstein. *et al*. 2000] (118)

CC is planar and rigid compound [119], white or pale yellow, odorless power, unstable in air and light, with a melting point of 116-113ºC. In commercially available preparations the isomers are in the ratio of 38% Zuclomiphene and 62% Enclomiphene [115].

1.6.2. Function

Clomiphene citrate acts to oppose the negative feedback effect of endogenous estrogens to increase gonadotropin secretion and stimulate ovulation. Most studies have found that clomiphene increases the amplitude of LH and FSH pulse, without change in pulse frequency. This suggests that the drug is acting largely at the pituitary level to block inhibitory action of estrogen on gonadotropin release from the gland [119]; Clomiphene citrate may also have a direct effect on the ovary, making the granulosa cell more sensitive to pituitary gonadotrophin [117].

Aims of the study

1. To measure and compare the levels of leptin, estradiol, vitamin E and selenium in PCOS patients and control group.
2. To study the relation between leptin and estradiol in HPG axis in PCOS patients.
3. To study the difference between groups of body mass index (BMI) in each parameter.
4. To study the role of each parameters in follicular phase, pre-ovulation, and luteal phase.

Chapter Three

Results and Discussion

3.1. Leptin

In this study, the results were given in tables (3.1& 3.2) and figure (3.2) showed a significant difference between follicular phase and Pre-ovulation (p<0.001) for Control, treated and untreated groups (p<0.05) and no significant difference was observed between pre-ovulation and luteal phase for each group in two studies (pool & follow up patients) and these results were agreed with Riad-Gabriel *et al*., 1998 who was found that leptin level increased throughout the time period of pre-ovulation. This was due to its release from mature ovarian follicles [(54)] while that level was decreased significantly in untreated group because of relatively elevated of leptin concentration in follicular phase and pre-ovulation of this group when compared it with other groups

Table (3.1): Difference in Leptin level among untreated, treated cases of PCOS and control groups during follicular, pre-ovulation and luteal phase (pool).

	Leptin (ng/ml) Untreated PCOS	Leptin (ng/ml) Treated PCOS	Leptin (ng/ml) Control	ANOVA
Cycle phase	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	
Follicular phase	6.1679±2.4558	5.1551±1.7268	4.2405±2.0710	**N.S.**
Pre-ovulation	9.8912±4.4254	8.1197±3.6839	8.0889±2.8953	**N.S.**
T-test	**P<0.05**	**P<0.001**	**P<0.001**	
Pre-ovulation	9.8912±4.4254	8.1197±3.6839	8.0889±2.8953	
Luteal phase	8.7258±3.1351	8.2028±3.4599	7.856±2.3487	**N.S.**
T-test	**N.S.**	**N.S.**	**N.S.**	

Table (3.2): Difference in Leptin level among untreated follow-up, treated follow-up cases of PCOS and control group during follicular, pre-ovulation and luteal phases.

	Leptin (ng/ml) Untreated PCOS follow-up	Leptin (ng/ml) Treated PCOS follow-up	Leptin (ng/ml) Control	ANOVA
Cycle phase	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	
Follicular phase	6.7228±2.5441	5.3560±1.3284	4.2405±2.0710	N.S.
Pre-ovulation	10.5716±3.9895	9.5430±2.5631	8.0889±2.8953	N.S.
T-test	P<0.05	P<0.001	P<0.001	
Pre-ovulation	10.5716±3.9895	9.5430±2.5631	8.0889±2.8953	
Luteal phase	9.1756±3.0793	9.1354±2.9066	7.856±2.3487	N.S.
T-test	N.S.	N.S.	N.S.	

which may play a role in arresting follicular development in certain women with PCOS [46].

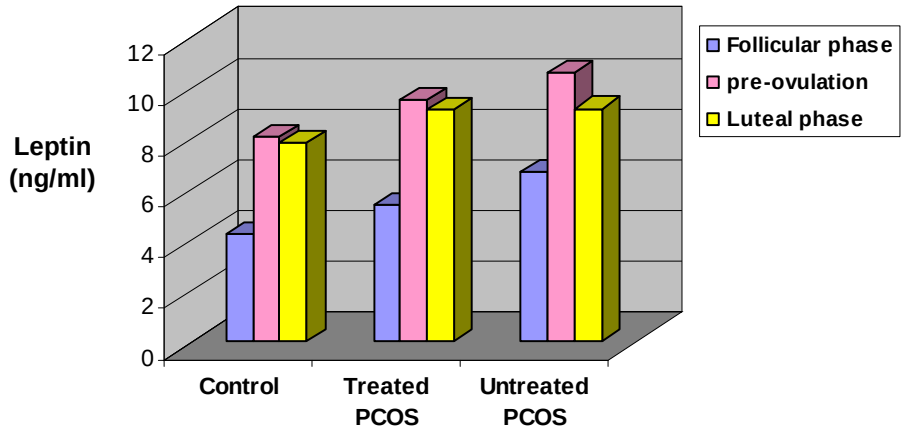


Fig.(3.1): Comparison of leptin levels during M.C. for each group.

According to other published studies in PCOS subject, serum leptin concentrations did not differ significantly between women with PCOS and BMI-matched control [59-62], as shown in tables (3.1, 3.2).

Also, there were no significant difference between patients groups and control group for certain BMI when regardless the M.C phases, as shown in table (3.3).

Table (3.3): Difference in Leptin level among untreated, treated and control group with body mass index.

		Leptin (ng/ml) Untreated PCOS		Leptin (ng/ml) Treated PCOS		Leptin (ng/ml) control	ANOVA
BMI (Kg/m^2)	No.	Mean ± SD	No.	Mean ± SD	No.	Mean ± SD	
<25	5	4.607±1.224	6	3.710± 0.261	5	3.2476±1.368	N.S
25-30	15	7.752±2.905	15	7.3417±2.603	14	7.0236±2.659	N.S.
>30	5	13.5013±1.826	5	10.7648±3.115	5	9.8118 ±2.551	N.S.
ANOVA		P<0.001		P<0.001		P<0.001	

There were highly significant difference (P<0.001), between leptin levels and different BMI for study group with positive correlation, as shown in table (3.3) & figure (3.2) & (3.3). and that agreed with Takeuchi and Tsutsumi, 2000 [122], Panidis *et al*., 2003 [123], Saleh *et al*., 2004 [124], Carmino *et al*., 2005 [62] and Hahn *et al*., 2006 [125], It may be the leptin was secreted from adipose tissues which inform the brain about the amount of energy stored in fat to reduce food intake, increase in energy expenditure and increased physical activity [27]

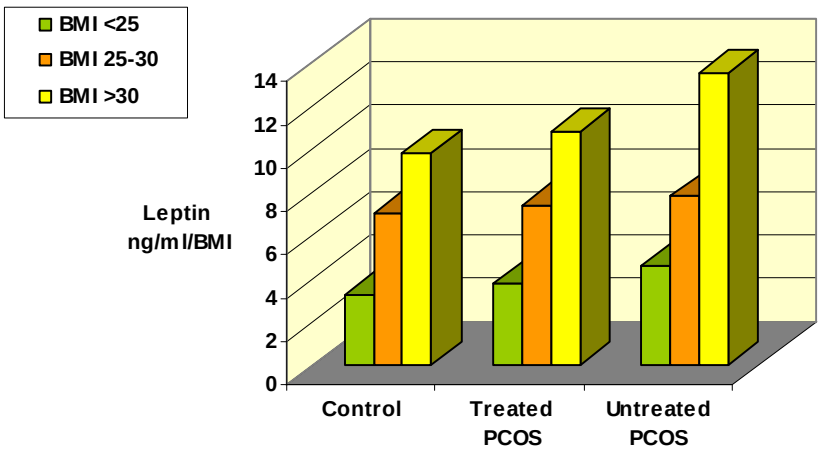


Fig.(3.2): Comparison of leptin levels with BMI for each group.

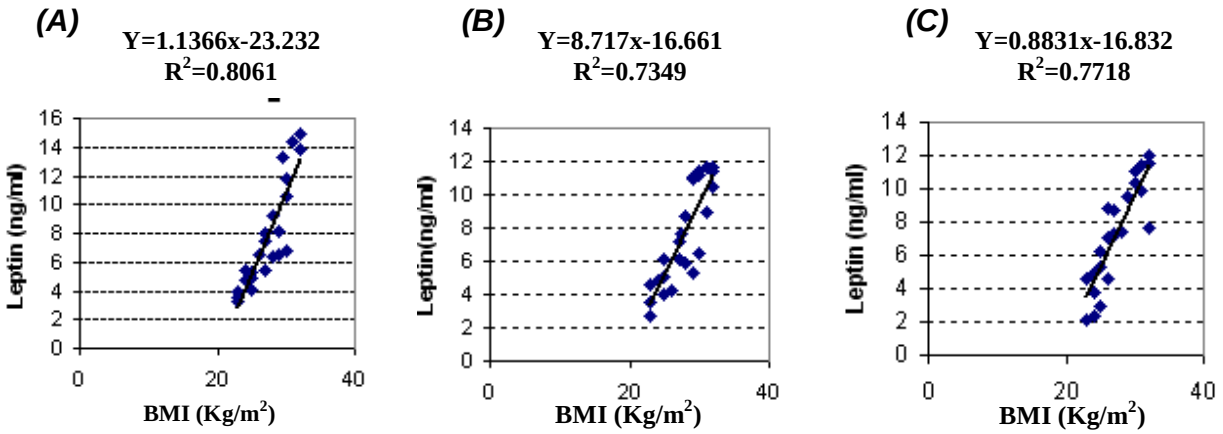


Fig. (3.3): Correlation between leptin concentration and BMI. (A) Correlation in Untreated PCOS (r=0.897), (B) Correlation in Treated PCOS (r= 0.857**), (C) Correlation in Control (r=0.878**).**
****Correlation is significant at 0.01 levels (2-tailed).**

3.2. Estradiol (E_2)

In present study, the results were found highly significant (p<0.0005) between follicular phase and pre-ovulation (Table 3.4 & 3.5) & (figure 3.4) and between pre-ovulation and luteal phase in control and treated groups because of estradiol in follicular phase is low and it release after follicular growth, reaching its peak before ovulation and then its level is decreased then return to arise in luteal phase [(11)].

Table (3.4): Difference in Estradiol level among untreated, treated cases of PCOS and control group during follicular, pre-ovulation and luteal phase (pool).

	Estradiol (pg/ml) Untreated PCOS	Estradiol (pg/ml) Treated PCOS	Estradiol (pg/ml) Control	ANOVA
Cycle phase	Mean ± SD	Mean ± SD	Mean ± SD	
Follicular phase	38.577±11.631	46.687±15.691	52.826±25.142	N.S.
Pre-ovulation	45.407±22.470	381.857±232.195	282.722±255.742	P<0.0005
T-test	P<0.05	P<0.0005	P<0.0005	
Pre-ovulation	45.407±22.470	381.857±232.195	282.722±255.742	
Luteal phase	57.238±15.128	91.468±27.785	83.445±32.123	N.S.
T-test	N.S.	P<0.0005	P<0.0005	

Table (3.5): Difference in Estradiol level among untreated follow-up, treated follow-up cases of PCOS and control group during follicular, pre-ovulation and luteal phase

	Estradiol (pg/ml) Untreated PCOS follow-up	Estradiol (pg/ml) Treated PCOS follow-up	Estradiol (pg/ml) Control	ANOVA
Cycle phase	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	
Follicular phase	37.428±9.554	42.600±13.50	52.826±25.142	**N.S**
Pre-ovulation	62.936± 7.240	487.400±41.932	282.722±255.742	**P<0.0005**
T-test	P<0.005	P<0.0005	P<0.0005	
Pre-ovulation	62.936± 7.240	487.400±41.932	282.722±255.742	
Luteal phase	67.296±5.800	80.822± 10.721	83.445±32.123	**N.S.**
T-test	N.S.	P<0.0005	P<0.0005	

While, Untreated group it was a significant difference (p<0.05) between follicular phase and pre-ovulation and no significant difference between pre-ovulation and luteal phase. Probably because estradiol was less secreted and follicular do not reach to maturation stage when estradiol at maximum level, for this reason that mean of serum estradiol in untreated is lower than others groups.

There were no significant difference between untreated, treated and control groups in follicular phase. Probably, the cause of follicular was in growth stages, while in pre-ovulation there was a significant different (p<0.0005), because of anovulatory follicles

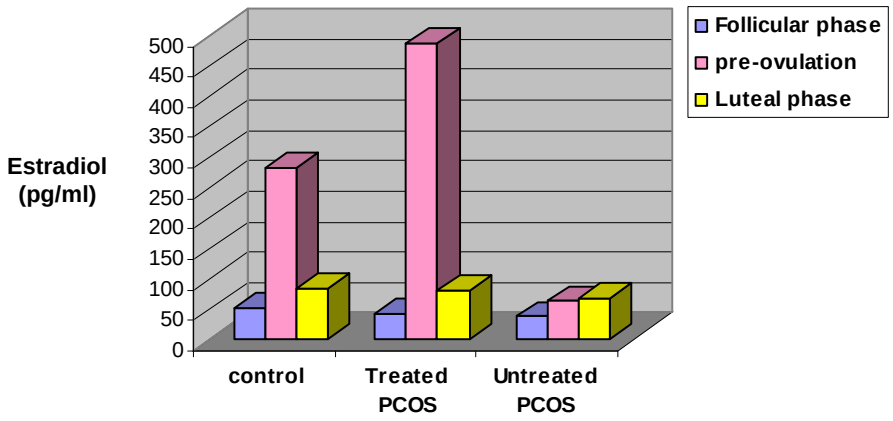


Fig.(3.4): Comparison of Estradiol levels during phases of M.C. for each group.

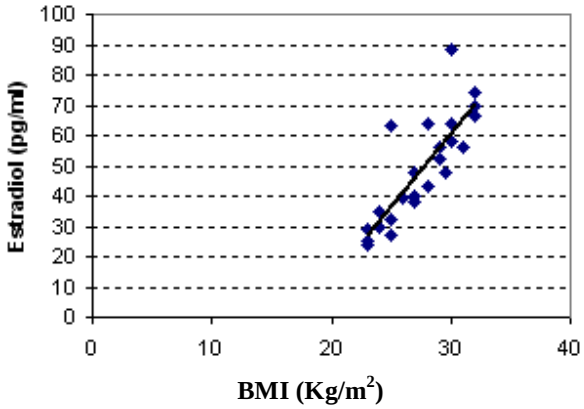
in untreated results from decreased activity of FSH-relatd to aromatase leading to inability of ovary to produce estradiol [18], for this reason the mean of serum estradiol level was less, when compared it with estradiol levels which was secreted by mature follicular in the other groups.

For this reason that there were significantly difference ($p< 0.05$) between studies groups and control group according to certain BMI. There were no significant differences between serum estradiol levels and different body mass index (BMI).

Table (3.6): Difference in Estradiol level among untreated, treated and control group with body mass index

		Estradiol (pg/ml) Untreated PCOS		Estradiol (pg/ml) Treated PCOS		Estradiol (pg/ml) Control	ANOVA
BMI(Kg/m^2)	No.	Mean ± SD	No.	Mean ± SD	No.	Mean ± SD	
<25	5	٣٤,٦٥٠± ٩,٤٢٢	6	102.353±31.416	5	187.004± 28.685	P<0.05
25-30	15	٤٩,٤٦٩±١١,٥٣٩	15	125.094±77.445	14	249.369±98.617	P<0.05
>30	5	٦٥,١٢٢± ٨,٢١٧	5	197.666±85.443	5	330.640±104.484	P<0.05
ANOVA		N.S.		N.S.		N.S.	

There are positive correlation significant appeared between BMI and Estradiol in Untreated PCOS, as shown in figure (3.5).



Y=4.8017x-83.146
R^2= 0.6807

Fig.(3.5): Correlation between Estradiol and BMI in Untreated PCOS(r=0.825).**
****Correlation significant at 0.01 levels (2-taied).**

This case appeared in Untreated PCOS group, it may be because adipose tissues considered an extra-ovarian aromatization for estradiol formation during ovarian suppression [13]. (**Future work** No.1)

Also there are positive correlation appeared between estradiol and leptin in pre-ovulation of PCOS groups and of control group, as shown in figure (3.6).

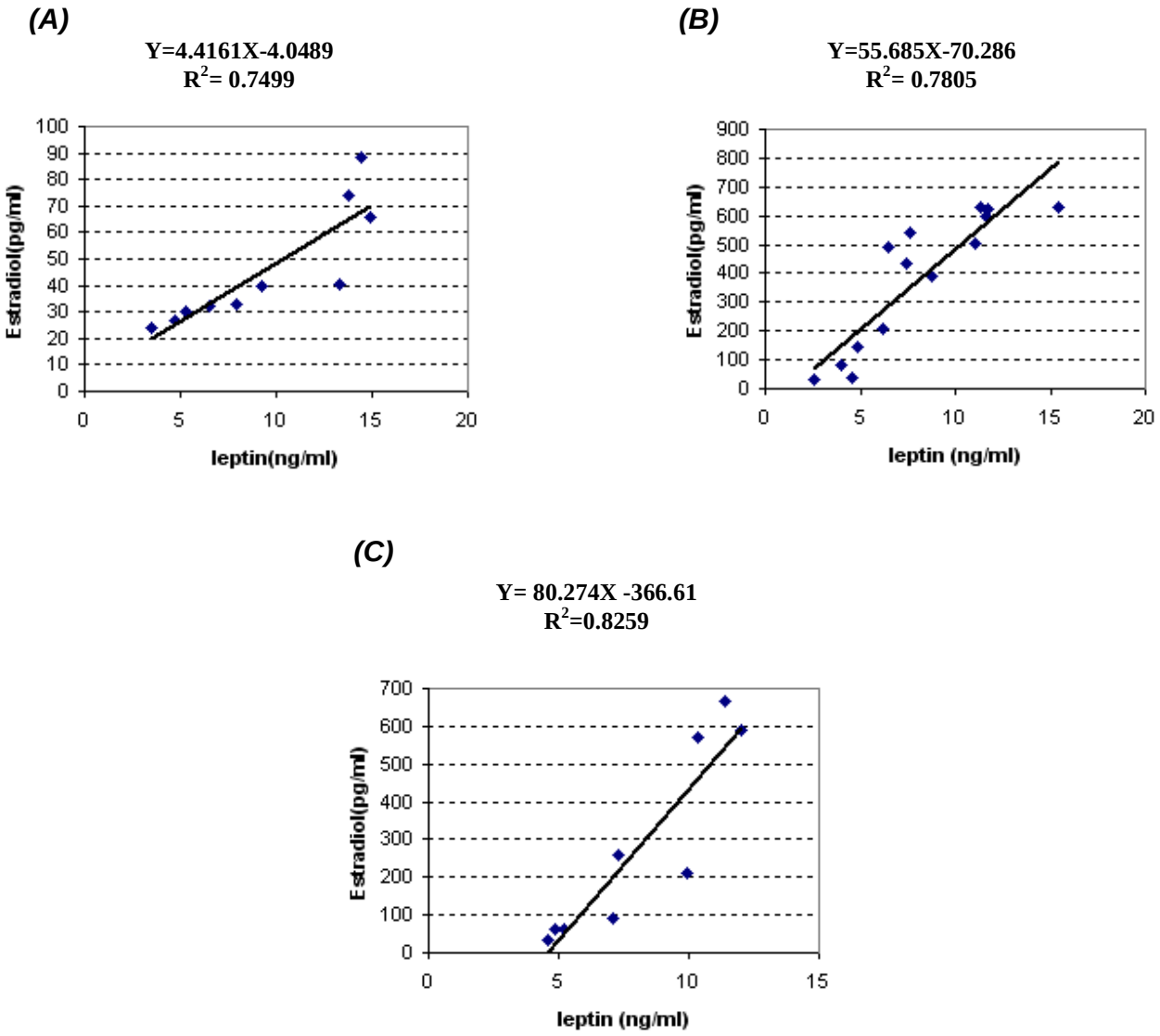


Fig. (3.6): Correlation between Leptin and estradiol. *(A)* In Untreated PCOS(r=0.865*), *(B)* In Treated PCOS (r=0.882*), *(C)* In Control (r=0.908*).

*** Correlation is significant at 0.05 levels (2-tailed).**

Because of estradiol can be an important regulator of leptin production in women and that agreed with Shimizu *et al.*, 1997[41], and Messinis *et al.*, 1998 [51]. While in PCOS patient, Probably, 1. because of correlation of estradiol with BMI and BMI with leptin leading to correlation between leptin and estradiol or 2.because of estradiol level is less that secret from ovary, which have positive feed back mechanism in adipose tissue leading to increasing secretion of leptin which have inhibitor effect on Neruopeptid Y (NPY) leading to increased Gonadotropin releasing hormone (GnRH) [31-36] (**Future work** No.2) and beside of that at Pituitary, leptin stimulated LH [39]. As show in figure (3.7).

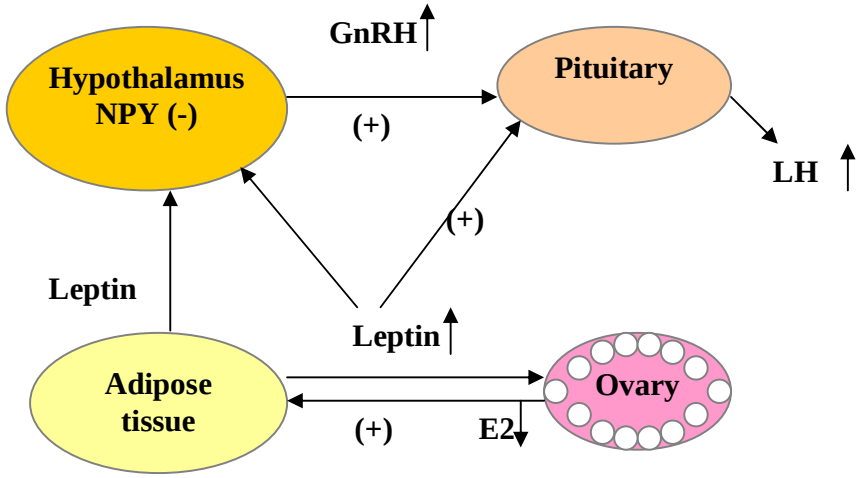


Fig.(3.7): Represent the correlation of leptin with H-P-O axis in PCOS.

3.3. Vitamin E

In the present study, the results were found in tables (3.7&3.8) & figure (3.8) significant difference (p<0.05) between follicular phase and pre-ovulation and this significant was disappeared between pre-ovulation and luteal phase for treated and control groups.

Table (3.7): Difference in Vitamin E level among untreated, treated cases of PCOS and control group during follicular, pre-ovulation and luteal phase (pool).

	Vit. E (µg/ml) Untreated PCOS	Vit. E (µg/ml) Treated PCOS	Vit. E (µg/ml) Control	ANOVA
Cycle phase	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	
Follicular phase	2.640±0.948	4.008±1.307	4.873±2.078	P<0.05
Pre-ovulation	3.261±.1.314	2.989±0.499	1.385±0.546	P<0.0005
T-test	N.S.	P<0.05	P<0.05	
Pre-ovulation	3.261±.1.314	2.989±0.499	1.385±0.546	
Luteal phase	4.011±1.032	3.379±2.176	3.948±1.874	N.S.
T-test	P<0.05	N.S.	N.S.	

Table (3.8): Difference in Vitamin E level among untreated follow-up, treated follow-up cases of PCOS and control group during follicular, pre-ovulation and luteal phase.

	Vit. E (µg/ml) Untreated PCOS follow-up	Vit. E (µg/ml) Treated PCOS follow-up	Vit. E (µg/ml) Control	ANOVA
Cycle phase	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	
Follicular phase	2.662± 0.643	3.498±1.307	4.873±2.078	P<0.05
Pre-ovulation	3.167±0.469	1.569±0.499	1.385±0.546	P<0.0005
T- test	N.S.	P<0.05	P<0.05	
Pre-ovulation	3.167±0.469	1.569± 0.499	1.385±0.546	
Luteal phase	4.252±0.481	2.676 ±2.176	3.948±1.874	N.S.
T-test	P<0.05	N.S.	N.S.	

In this study, the results were agreed with Rapoport *et al.*, 1998 [126] for decrease of Vitamin E level in pre-ovulation and luteal phase. It may be to balance the superoxide that generate from macrophages and neutrophiles, as they are present in ovary at ovulation and during corpus luteum regression [72-79] and because of these processes do

not occur in untreated group, there are no significant difference between follicular phase and pre-ovulation.

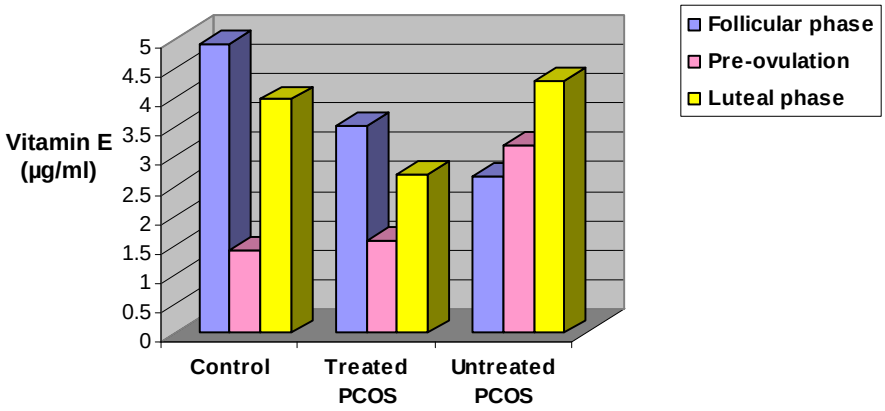


Fig.(3.8): Comparison of Vitamin E level during M.C. for each group.

For this reason, there were highly significant differences (p<0.0005) between studies groups and control group in pre-ovulation and there are significant difference (p<0.05) between groups studies in follicular phase with low level of vitamin E in untreated group, then increased the level in luteal phase leading to significant (p<0.05) between pre-ovulation and luteal phase of the same group, indicating that may be caused by oxidative stress at level of follicular causing to stop growing of follicular. (**Future work** No.3)

There are no significant difference between groups studies in certain BMI and between different of BMI in certain group, as show in table (3.9).

Table (3.9): Difference in Vitamin E level among untreated, treated and control group with body mass index.

		Vit. E (µg/ml) Untreated PCOS		Vit. E (µg/ml) Treated PCOS		Vit. E (µg/ml) Control	ANOVA
BMI (Kg/m^2)	No.	Mean ± SD	No.	Mean ± SD	No.	Mean ± SD	
<25	5	3.101±2.070	6	2.734±0.924	5	3.299±0.887	N.S.
25-30	15	3.710±1.380	15	2.160±1.628	14	3.984±2.025	N.S.
>30	5	3.741±0.578	5	3.964±3.398	5	5.947±1.993	N.S.
ANOVA		N.S.		N.S.		N.S.	

3.4. Selenium (Se)

In present study, the results were found significant differences (p<0.01) between follicular phase and pre-ovulation for treated and control groups, as shown in tables (3.10 &3.11). The results were agreed with Ha, and Smith, in 2003[127] , from the lowest level during the follicular phase to a maximum level during pre-ovulation, which coincided with elevated of 17-β-estradiol, as Shown in figure (3.9).

Table (3.10): Difference in Selenium level among untreated, treated cases of PCOS and control group during follicular, pre-ovulation and luteal phase (pool).

	Se (ng/ml) Untreated PCOS	Se (ng/ml) Treated PCOS	Se (ng/ml) Control	ANOVA
Cycle phase	Mean ± SD	Mean ± SD	Mean ± SD	
Follicular phase	69.857± 7.603	77.333±3.204	98.500±5.781	P<0.000005
Pre-ovulation	75.300± 10.521	90.923±7.857	109.222±8.814	P<0.000005
T-test	N.S.	P<0.01	P<0.01	
Pre-ovulation	75.300± 10.521	90.923±7.857	109.222±8.814	
Luteal phase	72.125±6.854	88.333±6.918	104.000±9.712	P<0.000005
T-test	N.S.	N.S.	N.S.	

Table (3.11): Difference in Selenium level among untreated follow-up, treated follow-up cases of PCOS and control group during follicular, pre-ovulation and luteal phase.

	Se (ng/ml) Untreated PCOS follow-up	Se (ng/ml) Treated PCOS follow-up	Se (ng/ml) Control	ANOVA
Cycle phase	Mean ± SD	Mean ± SD	Mean ± SD	
Follicular phase	66.000±4.636	78.2000±4.024	98.500±5.781	P<0.000005
Pre-ovulation	76.000±5.744	93.000±5.831	109.222±8.814	P<0.000005
T-test	P<0.01	P<0.005	P<0.01	
Pre-ovulation	76.000±5.744	93.000±5.831	109.222±8.814	
Luteal phase	71.000±2.000	86.200±5.069	104.000±9.712	P<0.000005
T-test	N.S.	N.S.	N.S.	

Ohwada *et al*., 1996, found that endometrial GPx activity in women is stimulated by estrogen and that uterine GPx activity in spayed rats can be elevated by exogenous estradiol treatment [128]. Massafra *et al*., 1997, have suggested that estradiol affects the maturation of bone marrow to stimulate the synthesis of active GPx [129]. From those studies, I conclude that estrogens have indirect effect on selenium by GPx. And that effect appeared highly significant (p<0.000005) between studies groups while the significant (p<0.01) that appeared between follicular phase and pre-ovulation with lower level of selenium concentration in untreated PCOS, as shown in figure (3.12). It may be because of oxidative stress is happened. (**Future work** No.3)

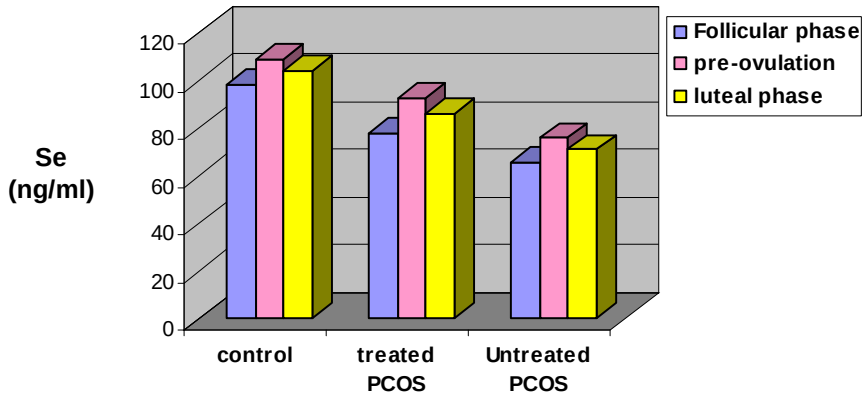


Fig.(3.9): Comparison of Selenium levels during M.C. for each group.

But that effect is no significant in untreated group between follicular phase and pre-ovulation in pool study because of plasma GPx has been shown to 10% to 15% of the plasma selenium [130] and beside of this the different in metabolism and genetics from person to another causing to disappear the significant. (**Future work** No.4)

For the same reason which was mentioned above, there were strongly significantly different (p<0.005) between studies groups in certain BMI, but no significant in different BMI for one group.

Table (3.12): Difference in Selenium level among untreated, treated and control group with body mass index.

		Se (ng/ml) Untreated PCOS		Se (ng/ml) Treated PCOS		Se (ng/ml) Control	ANOVA
BMI (Kg/m^2)		Mean ± SD		Mean ± SD		Mean ± SD	
<25	5	73.800±7.429	6	88.000±10.020	5	102.200±2.588	P<0.005
25-30	15	72.066 ±4.817	15	88.875±8.285	14	104.461±11.892	P<0.005
>30	5	72.444±12.299	5	81.333±16.669	5	102.833±9.724	P<0.005
ANOVA		N.S.		N.S.		N.S.	

Conclusions

Data showed:-

1. Leptin concentrations in women with PCOS are similar to BMI-matched controls, with strongly associated between leptin and BMI in each group.
2. Elevations of leptin in pre-ovulation indicating that it have a role in follicular growth and have relation with estradiol.
3. Vitamin E have role in ovulation.
4. Selenium and Vitamin E have lowered significantly in untreated PCOS, suspecting the oxidative stress was occurred.
5. No correlation between Selenium and Vitamin E with BMI.

Future Work

1. Measure 17β-hydrosteriododehydrogenase (17β-HSD) type 1 activity in adipose tissue of PCOS.
2. Measure the degree of inhibition for NPY.
3. Measure superoxide in follicular fluid and study of its relation with antioxidant of the cystic in follicular phase of PCOS.
4. Measure of GPx in follicular fluid.

Chapter Two

Materials and Methods

2.1. Subjects Selection

The control cases and analytic study conducted at College of Science / Chemistry Department and College of Medicine at Al-Nahrain University Hospital, Department of Obstetric and Gynecology and IVF Institute of Embryo Research and Infertility Treatment throughout the period from Dec., 20th 2006 to Apr., 15th, 2007.

Seventy-five women were included in this study, the study was consisted from three groups and each group was subdivided in three groups according to their phases of menstrual cycle.

The first two groups were included fifty-one patients diagnosed clinically and biochemically for PCOS patients.

The first group included twenty-five patients untreated with clomiphene citrate and the second group included twenty-six patients were treated by clomiphene citrate. As show in figure (2.1).

In each two groups only five patients were followed up in the clinics to monitor them throughout phases of menstrual cycle, and other patients where classified in pool study in order they have the same BMI throughout phases of menstrual cycle.

Twenty-four patients, age and body mass index matched normal women were used as control group, they were not taking any drugs and their ages were reproductive women age.

Detailed clinical assessment was done by the gynecologist regarding to history of infertility and its drugs treatment, menstruation, height and body weight, hair

distribution, thyroid, breast, blood pressure examination and history of family also ovarian size, number of cystic and texture were assessed by ultrasound examination (Appendix 1).

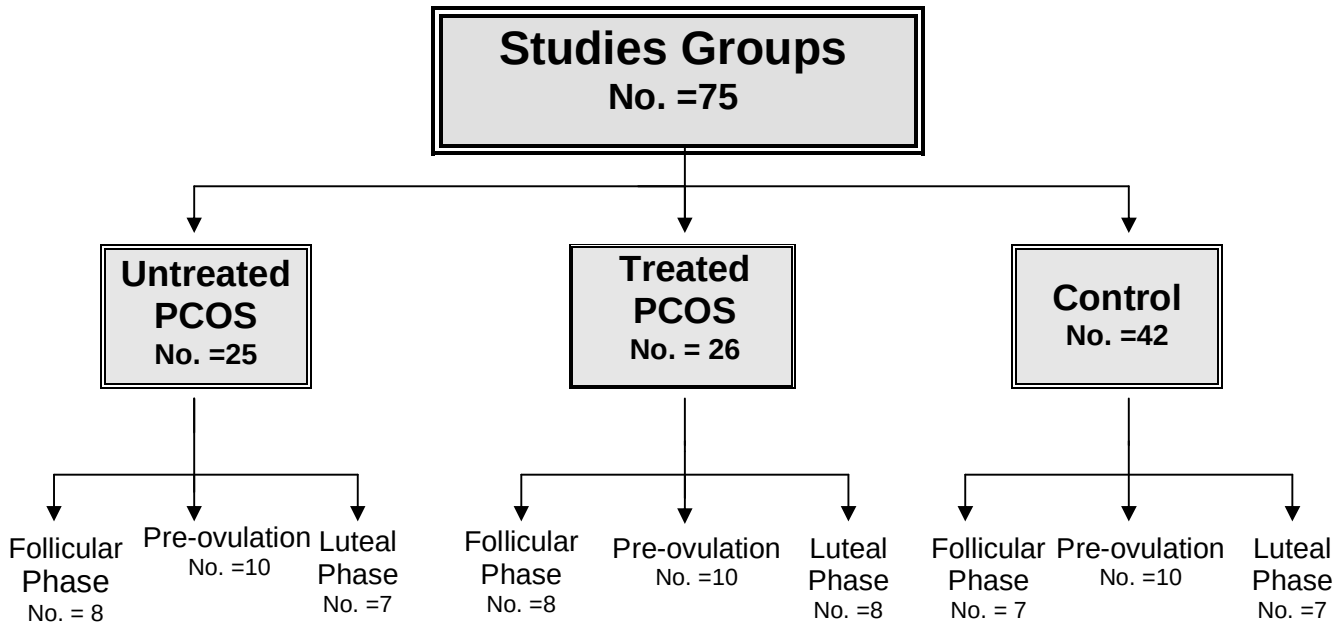


Fig. (2.1): Studies groups.

2.2. Serum collection

Blood was collected at each phases of menstrual cycle, follicular phase, 1-9 days, pre-ovulation, 10-15days and luteal phase, over 15 days to measure all parameters. Blood was drawn from fasting patients by disposable plastic syringes. The serum was separated after 30 min of blood clotting in test tubes by centrifugation at 2000 rpm for 10 minute. at room temperature. The serum was stored in polyethylene tube at – 20˚C until assayed.

2.3. Chemicals and Reagents

2.3.1. Hormones:

A. Leptin (Sandwich ELISA) kits: by DRG Manufacturer, Germany.

1. Microtiter wells: wells coated with anti-leptin antibody (monoclonal)
2. Standard concentrations (0, 2, 5, 25, 50 and 100 ng/ml), Control (2 levels, low and high) and assay buffer contain 0.3% proclin as preservative.
3. Antiserum (polyclonal leptin antiserum) contains 0.3% proclin as preservation.
4. Enzyme Complex: Anti-rabbit complex conjugated to horseradish peroxidase, contains 0.3% proclin.
5. Substrate solution: TMB.
6. Stop solution contain 0.5M H_2SO_4.
7. Wash solution.

B. VIDAS Estradiol II (E2) Kits: by bioMérieux, France.

1. Interior of Solid Phase Receptacles (SPRs) coated with polyclonal anti-estradiol immunoglobulin (rabbit).
2. E_2 II Control: Human serum, 17 β estradiol and 1 g/l sodium azide.
3. E_2 II Calibrator: Human serum, 17β estradiol and 1g/l sodium azide.
4. Strip: consist of 10 wells

   First: sample well.

   Second, third & fourth: Empty wells.

   Fifth: containing conjugate: alkaline phosphatase labeled estradiol derivative + 0.9 g/l sodium azide (400 µl).

   Sixth: Empty well.

   Seventh & Eight: Wash buffer: Tris-NaCl (0.05 mol/l) PH 9 +1 g/l sodium azide.

Ninth: Wash buffer: diethanolamine (DEA) (1.1 mol/l, or 11.5 %, pH 9.8) and 1 g/l sodium azide.

Tenth: Cuvette with substrate: 4-Methyl-umbellifryl phosphate (0.6nmol/l) +diethanolamine (0.62 mol/l, or 6.6 %, pH 9.2) and 1g/l sodium azide.

2.3.2. Vitamin E:

Vitamin E standard, 5-sulphosalicylic acid, Methanol for (HPLC grade) and Distill water.

2.3.3. Selenium

The standards of Selenium were prepared in Ministry of Science and Technology.

2.4. Instruments:

2.4.1. Enzyme-linked immunosorbent assay (ELISA)

ELISA-Kit based on the sandwich principle.

The microtiter wells are coated with a monoclonal antibody directed towards a unique antigenic site on a leptin molecule.

An aliquot of patient sample containing endogenous leptin is incubated in the coated well with a specific rabbit anti leptin antibody. A sandwich complex is formed. After incubation the unbound material is wash off and an anti rabbit peroxidase conjugate is added for detection of the bound leptin, added the substrate solutions the intensity of violet color developed is proportional to the concentration of leptin in the patient sample.

2.4.2. Mini Vidas

The assay principle combines a competitive method with a final fluorescent detection (ELFA) Enzyme linked fluorescent assay.

The Solid Phase Receptacle (SPR) serves as the solid phase as well as the pipetting device for the assay.

Reagents for the assay are ready-to-use and pre-dispensed in the sealed reagent strips. All of the assay steps are performed automatically by the Mini Vidas instrument. The reaction medium is cycled in and out of the SPR several times.

The sample is transferred into well containing the conjugate, which is an alkaline phosphatase labeled estradiol derivative

The estradiol present in the serum and the estradiol derivative in the conjugate compete for the anti-estradiol specific antibody sites coated to the inner surface of the SPR. Unbound components are eliminated during the washing steps

During the final detection step, the substrate (4-methyl-umbelliferyl phosphate) is cycled in and out of the SPR.

The conjugate enzyme catalyzes the hydrolysis of this substrate into a fluorescent product (4-methyl umbelliferone), the fluorescence of which is measured at 450 nm.

The intensity of the fluorescence is inversely proportional to the concentration of antigen present in the sample.

2.4.3. High Performance Liquid Chromatography (HPLC)

HPLC is a process by which a mixture of compounds is separated into its constituent compounds by flowing a liquid (i.e. mobile phase) over a stationary phase [120].

HPLC 10AVP is consisting from six instruments were made in Shimadzu (Kyoto, Japan):

a. De-gazer unite:

DGU-12A was used to prevent the babbles.

b. Solvent reservoir:

The solvent reservoir is usually a flask that contain mobile phase.

c. Pump:

The LC-8A pump drives the mobile phase from the reservoir and through the injector, the column, and detector.

The mode of pump was used is isocratic mode, which the mobile phase composition remains constant through out the chromatographic run.

d. Injector:

An aliquot of sample is introduced into a liquid chromatograph via some type of sample injector. In this study, (SIL-10A Shimadzu Auto Injector) was automated inject 20 µl and consequently multisampling loaders that incorporate a loop injector.

e. Column:

The chromatographic column (stationary phase) is Octa Decayl Silica C-18 column (ODS-Column) (250×4.6mm i.d) made in Germany which consists of a stainless steel tube that contain a packing with 5µm particle size.

f. Detector:

SPD-M10A shimadzu diode array detector was used to measure the absorption of radiant energy by vitamin E at wavelength 292 nm as they elute from the chromatographic column.

g. Data recorder:

The time required for a compound to pass through a liquid chromatograph is called the retention time, where the peak area was drawn by computer which is

proportion to concentration of Vitamin E in the serum. The computer was connect with HPLC system via in shimadzu class-vp5 chromatography data system program supplied by the manufacture; Epson LQ-300 printer model P852A (Japan).

These entire instruments were controlled by SCL-10Avp Shimadzu system controller.

2.4.4. Atomic Absorption Spectrophotometry (AAS)

The principle of atomic absorption measurements is the amount of light at the resonant wavelength which is absorbed as the light passes through a cloud of atoms. As the number of atoms in the light path increases, the amount of light absorbed increases in a predictable way. By measuring the amount of light absorbed, a quantitative determination of the amount of analyte element present can be made. The use of special light sources and careful selection of wavelength allow the specific quantitative determination of individual elements in the presence of others.

In the present study, flameless atomic absorption was used for determination of selenium in serum

Graphite furnace atomic absorption

By far the most advanced and widely used high sensitivity sampling technique for atomic absorption is the graphite furnace. In this technique, a tube of graphite is located in the sample compartment of the AA spectrometer, with the light path passing through it. A small volume of sample solution is quantitatively placed into the tube, normally through a sample injection hole located in the center of the tube wall. The tube is heated through a programmed temperature sequence until finally the analyte present in the sample is dissociated into atoms and atomic absorption occurs.

As atoms are created and diffuse out of the tube, the absorbance rises and falls in a peak-shaped signal. The peak height or integrated peak area is used as the analytical signal for quantitation [(121)].

The instruments used to determine selenium in the present study was:

Perkin-Elmer (USA) AA model 305:

Determination of selenium was performed using Perkin-Elmer model 305 AAS equipped with a heated graphite furnace model (HGA) 2200. Argon gas (99.999% purity) was used as a purge gas at flow rate of 50 ml/min. Argon gas flow was maintained continuously through during and charring steps and gas stop mode was used during atomization stage. As-1 autosampler was used for dispensing samples into the graphite furnace. Instrumental setting and working condition for determination of Se is given in table (2.1).

Table (2.1): Instrumental Parameter for perkin Elmer 305 Flameless AAS for selenium.

Parameters	Se
Wavelength (λ)	196 nm
Lamp current	16 mA
Volume of sample	10µl
Sheathing gas	Argon
Drying time	30 min.
Ashing temp.	1000ºC
Atomization temp	2000ºC
Atomization time	3 sec.

2.5. Analysis of sample

2.5.1. Leptin

1. The desired number of microtiter wells was secured in the holder.
2. 15µl of each standard, controls and samples were dispensed.
3. 100 µl of Assay Buffer were dispensed into each well.
4. For 10 seconds was mixed thoroughly. It is important to have a complete mixing in this step.
5. For 120 minutes at room temperature was incubated without covering the plate.
6. The contents of the wells were shaken out briskly.
7. The wells were rinsing 3 times with diluted Wash Solution (300 µl per well) and the wells were stroked sharply on absorbent paper to remove residual droplets.
8. 100 µl of Antiserum was added to each well.
9. For 30 minutes at room temperature was incubated.
10. The contents of the wells were shaken out briskly.
11. The wells were rinsing 3 times with diluted Wash Solution (300 µl per well) and the wells were stroked sharply on absorbent paper to remove residual droplets.
12. 100 µl of Enzyme Complex was dispensed into each well.
13. For 30 minutes at room temperature was incubated.
14. The contents of the wells were shaken out briskly.
15. The wells were rinsing 3 times with diluted Wash Solution (300 µl per well) and the wells were stroked sharply on absorbent paper to remove residual droplets.
16. 100 µl of Substrate Solution was added to each well.
17. For 15 minutes at room temperature were incubated.

18. 50µl of Stop Solution was added to each well for stop the enzymatic reaction.

19. The Optical density was read at 450±10 nm with a microtiter plate reader within 10 minutes after adding the Stop Solution.

20. Standard curve was drawn by using logistics fit curve MPM program. As show below in figure (2.2).

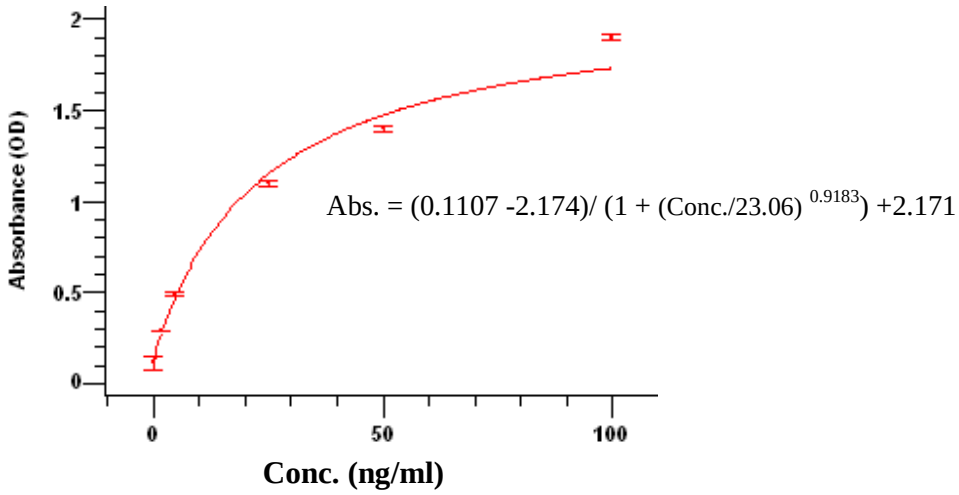


Fig. (2.2): Standard Curve of leptin concentrations.

2.5.2. Estradiol II (E2)

1. The required reagents were removed from the refrigerator and allow them to come up to room temperature for 30 minutes before use.

2. The calibrator, control and samples were mixed with a vortex type mixer in order to improve result reproducibility.

3. 200 µl of sample, E_2 II calibrator or control were pipette into sample well.

4. The SPRs and Reagent strips were inserted into instrument in the positions indicated on the screen.

5. All the assay steps were performed automatically by the mini vidas instrument which was completed within 60 minutes.

2.5.3. Vitamin E

A. Standards preparation

Stock Standard was prepared in methanol at concentration 25 ppm and 15, 10, and 5 ppm standards used for calibration during quantitative analysis were prepared by diluting one from the other with methanol. As show below in figure (2.3)

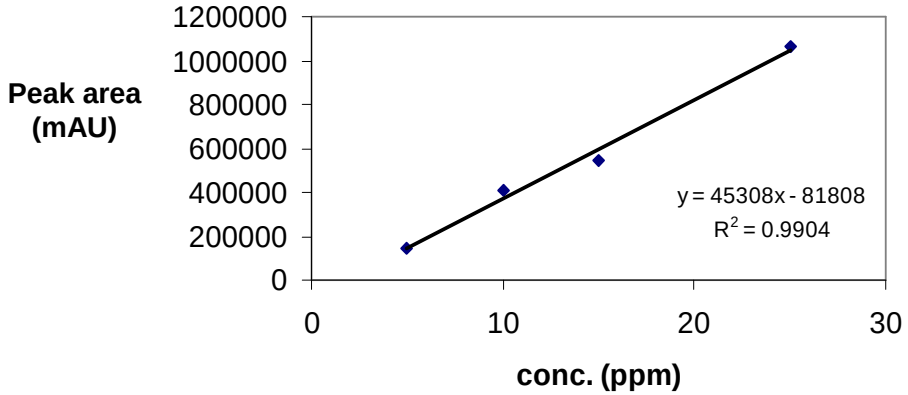
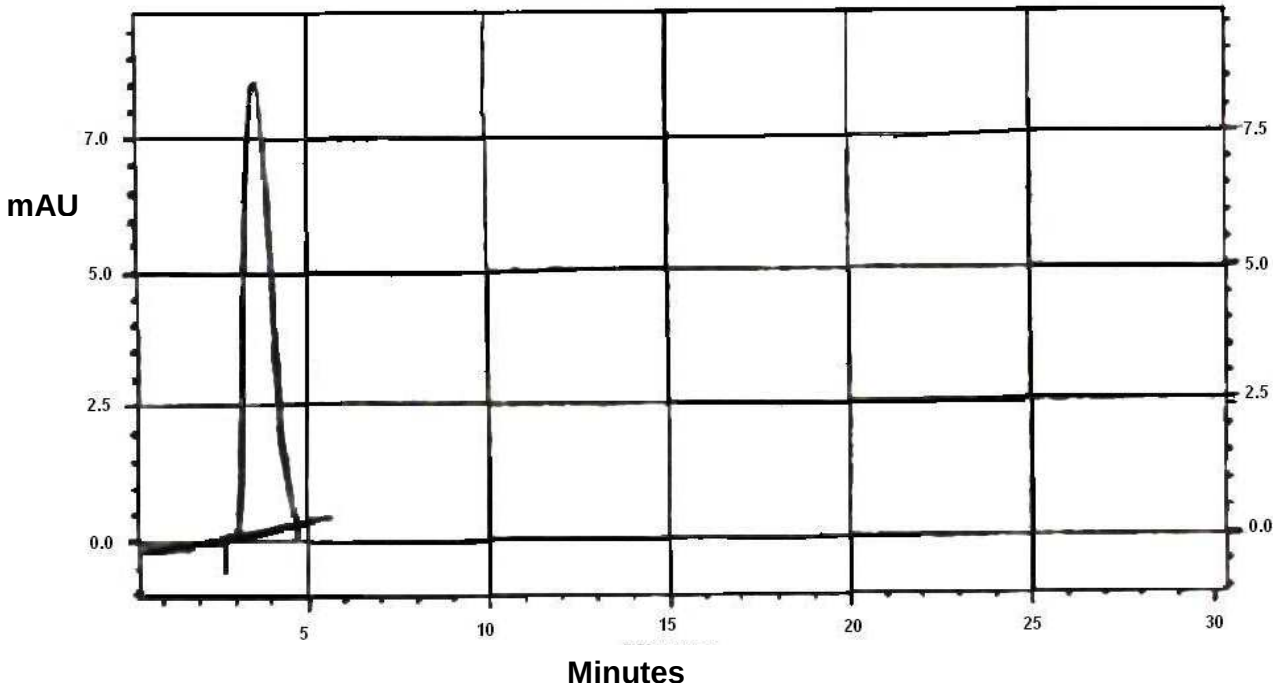
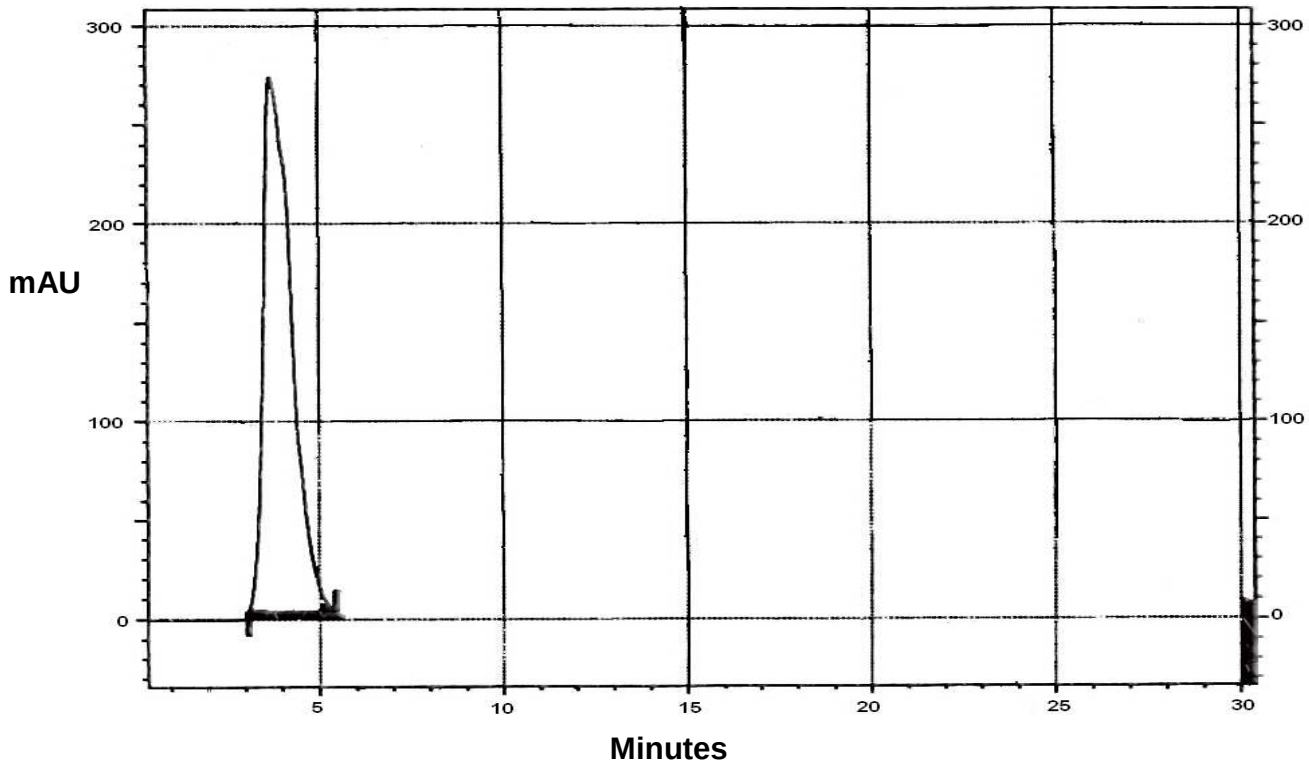


Fig. (2.3): Standard Curve of Vitamin E concentrations.



(A)



(B)

Fig. (2.4): The HPLC analysis of Vitamin E. (A) Standard 10 ppm, (B) Sample of Untreated PCOS. By using C-18 ODS column (250* 4.6mm Id), the mobile phase was methanol distilled water (90:10 V/V), flow rate 1ml/min., absorbance at 292nm, Retention time: 3.٦٨ min.

B. Sample preparation

Four hundred microliter (400 µl) of frozen serum after completed thawing, deproteinized by adding 50 µl of (15%) 5-sulphosalicylic acid, mixed and centrifuged at 3000 rpm for 10 minutes. Then take the filtered, diluted ten folds with deionized water and filtrated by mini pore.

C. Mobile Phase preparation

The mobile phase was prepared by methanol: distilled water (90:10 V/V) by using the same HPLC as described in (2.4.3).

2.5.4. Selenium

1. Stock standard of the 100 ppm was prepared and from it prepared the standards 0.0, 0.005, 0.01, 0.04, 0.07 and 0.1ppm sequentially from most concentrate to most dilute and analyzed sequentially from most dilute to most concentrate.
2. Serum samples were thawed and diluted two folds with deionized water.
3. Instrumental and gas flow settings and aspiration rate for selenium are given in table (2.1) Aspiration of zero concentration standard was done to set baseline to read zero absorbance; this process was repeated frequently to correct baseline drift.

2.6. Statistical analysis:

Statistical analysis was performed using SPSS for windows TM version 10and Microsoft EXCEL for windows 2003 &2007.

- **Descriptive statistics:**
  a. Statistical tables.
  b. Arithmetic mean.
  c. Standard deviation.

- **Differential statistics:**
  a. T-test**.**
  b. ANOVA test**.**
  c. The correlation coefficient**.**

Abbreviations

AAS	Atomic Absorption Spectrophotometry.
ANOVA	Analysis of variance.
BMI	Body Mass Index.
CC	Clomiphene citrate.
E2	Estradiol.
ELISA	Enzyme-linked immunosorbent assay
FSH	Follicular stimulating Hormone.
GPx	Glutathione peroxidase.
HPLC	High Performance Liquid Chromatography.
Id	Internal dimeter.
KDa	Kilo Dalton unit.
LH	Luteinizing hormone.
M.C.	Menstrual Cycle.
mAU	Milli Ampare Unit
NPY	Neruopeptide Y.
N.S.	No Significant.
Ob	Obesity gene.
Ob-Rs	Leptin receptors.
ODS	Octa Decyl Silane.
PCOS	Polycystic Ovary Syndrome.
ppm	Part per million.
P value	Probability level of statistical decision.
ROS	Reactive Oxygen Species.
r.p.m	Round per minute.
SD	Standard Deviation
Se	Selenium.
SHBG	Sex-Hormone Binding Globulin.
SPR	Solid Phase Receptable.
T- test	Tukey test.
Vit.	Vitamin

Contents

Contents

List of Tables

List of Figures

Contents

ARepublic Of Iraq
The Ministry Of Higher Education
And Scientific Research
Al-Nahrain University
College Of Science
Department Of Chemistry

A Study of Relationship between Leptin with Estradiol, Antioxidants (Selenium and Vitamin E) in the Polycystic Ovary Syndrome Patients

A Thesis

Submitted to the College of Science at Al-Nahrain University

In Partial Fulfillment of the Requirements for the degree of Master of Science

in

BIOCHEMISTRY

By

Farah Aqeel Rasheed
B.Sc. in Chemistry
Al-Nahrain University
(2005)

November 2007 **Thee Alquada 1428**

بسم الله الرحمن الرحيم

﴿وَنَزَعْنَا مَا فِي صُدُورِهِم مِّنْ غِلٍّ تَجْرِي مِن تَحْتِهِمُ الأَنْهَارُ وَقَالُواْ الْحَمْدُ لِلّهِ الَّذِي هَدَانَا لِهَـذَا وَمَا كُنَّا لِنَهْتَدِيَ لَوْلا أَنْ هَدَانَا اللّهُ لَقَدْ جَاءتْ رُسُلُ رَبِّنَا بِالْحَقِّ وَنُودُواْ أَن تِلْكُمُ الْجَنَّةُ أُورِثْتُمُوهَا بِمَا كُنتُمْ تَعْمَلُونَ ﴾

صدق الله العظيم
الاعراف(٤٣)

Committee Examine

We the examining committees, certify that we have read this thesis & examined the student in it's contents and that, according to our opinion, is accepted as a thesis for the degree of master of science in Biochemistry with grade (Excellent).

Signature:
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Member
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Name: *Lect. Dr. Sawsan H. Authman.*
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Name:*Ass. Prof.Dr.Salman A.Ahmad.*
Member / Advisor
Date:

Signature:
Name: *Prof.Dr.Abdul Wahab R.Hamad.*
Member / Advisor
Date:

I here by certify upon the decision of the examining committee.

Signature:
Name: Assist. Prof. LAITH ABDUL AZIZ AL-ANI
Address: Dean of the College of Science
Date:

Supervisor Certification

We certify that this thesis was prepared under our supervision at the Department of Chemistry, College of science, Al-Nahrain University as a partial requirements for the Degree of M.Sc in Chemistry.

Signature:
Name:*Ass. Prof. Salman A. Ahmad.*
Dept. of Chemistry,
College of Science,
Al-Nahrain University.

Signature:
Name: *Prof.Abdul Wahab R. Hamad.*
Dept. of Chemistry & Biochemistry,
College of Medicine,
Al-Nahrain University.

In view of the available recommendation, I forward this thesis for debate by the examining committee.

Signature:
Name:*Dr. Salman A. Ahmad.*
Head of the Department of Chemistry,
College of Science,
Al-Nahrain University.

To Candles of My Life

My Family

To Inspiring Influence

Dr. Tariq Muhsin Haider

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Farah

CHAPTER 1

Introduction & Literature review

CHAPTER 2

Material & Methods

CHAPTER 3

Results & Discussion

References

Appendixes

Appendix 1

Questionnaire

Tube No. :

Name :

Age :

Length :
Weight :
BMI :

Cycle day:

Treated :

Ultrasound:

FSH : LH :

Prolactin :(Normal)

Thyroid function :(Normal)

Leptin:

Estradiol (E2):

Selenium (Se):

Vitamin E:

Appendix 2

Relations in this study

BMI (kg/m^2)				
	Untreated PCOS	**Treated PCOS**	**Control**	**Page**
Leptin (ng/ml)	**0.897****	**0.857****	**0.878****	37
Estradiol (E_2) (pg/ml)	**0.825****	-	-	39
Leptin(ng/ml)				
	Untreated PCOS	**Treated PCOS**	**Control**	**Page**
Estradiol (E_2) (pg/ml)	**0.865***	**0.882***	**0.908***	40

*** Correlation is significant at 0.05 levels (2-tailed).**

****Correlation significant at 0.01 levels (2-taied).**

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين \كلية العلوم
قسم الكيمياء

دراسة العلاقة بين اللبتين مع الاسترادايول و مضادات الاكسدة(السيلينوم و فيتامين إي) في مرضى المبيض المتعدد الأكياس

رسالة مقدمة

الى

كلية العلوم جامعة النهرين

كاستكمال جزئي لمتطلبات نيل درجة ماجستير

علوم في الكيمياء الحياتية

من قبل

فرح عقيل رشيد

بكلوريوس علوم كيمياء (جامعة النهرين ٢٠٠٥)

ذي القعدة ١٤٢٨

تشرين الثاني٢٠٠٧

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